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14. ABSTRACT

Defined by the presence of malignant cells spreading within intact prostatic ducts and acini, intraductal carcinoma of the prostate (IDC-P) occurs almost exclusively in high Gleason grade and stage tumors and is a consistent independent risk factor for tumor progression and death. Importantly, however, IDC-P is currently systematically under-diagnosed in needle biopsies because it has significant morphologic overlap with another intraepithelial lesion, high grade prostatic intraepithelial neoplasia (HGPIN). We hypothesize that IDC-P represents progression of invasive high-risk prostate carcinoma, and thus is molecularly distinct from HGPIN. Herein, we have conducted an unbiased, three-pronged set of experiments to definitively identify the molecular signature of IDC-P using a combination of protein, RNA, and DNA-based assays.

15. SUBJECT TERMS
Prostate cancer, intraductal carcinoma, molecular profiling

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1. INTRODUCTION:

Although non-invasive, intraductal carcinoma of the prostate (IDC-P) has long been recognized by pathologists as an extremely high risk feature. Defined by the presence of malignant cells spreading within intact prostatic ducts and acini, IDC-P occurs almost exclusively in high Gleason grade and stage tumors and is a consistent independent risk factor for tumor progression and death in cohorts treated with surgery or radiotherapy. Importantly, however, IDC-P is currently systematically under-diagnosed in needle biopsies because it has significant morphologic overlap with another intraepithelial lesion, high grade prostatic intraepithelial neoplasia (HGPIN). Since HGPIN is a morphologically similar lesion with virtually no prognostic significance, ***we propose that the systematic under-diagnosis of IDC-P in needle biopsies results in the under-recognition of potentially aggressive prostate tumors***. We have found that IDC-P and HGPIN may be readily separable at the *molecular* level, as IDC-P shows an extremely high rate of PTEN loss (84%), a rate even exceeding that seen in invasive high Gleason grade tumors. In contrast, HGPIN never shows loss of this tumor suppressor. Although our preliminary candidate gene data is compelling, *the current challenge is to systematically elucidate the molecular profile of IDC-P, a study which will not only yield additional clinically useful markers of this specific lesion but also elucidate the molecular features of an extremely high risk subset of prostate tumors*. The aims of the current study were to: 1) *Validate* PTEN and ERG as specific, clinically applicable markers of IDC-P, using a combination of immunohistochemistry and fluorescence *in situ* hybridization (FISH); 2) *Profile* the gene expression signature of IDC-P and systematically compare it to HGPIN, identifying additional candidate markers for distinguishing the two lesions; and 3) *Integrate* IDC-P into the molecular landscape of invasive carcinoma, both at the gene expression and genomic levels, using a combination of bioinformatics, targeted next generation sequencing and copy number variation analysis.

2. KEYWORDS:

Prostatic carcinoma, intraductal carcinoma, high grade prostatic intraepithelial neoplasia, molecular profiling

3. ACCOMPLISHMENTS:

a. What were the major goals of the project?

Task 1: *Validate* PTEN and ERG as specific molecular markers of IDC-P (months 4-24, allowing for 3 month regulatory review of IRB protocols). 1a. Assess PTEN/ERG protein status via immunohistochemistry (IHC) in 40 biopsies each of: isolated IDC-P meeting current morphologic criteria, IDC-P with concurrent invasive carcinoma, and age-matched cases of isolated HGPIN (months 4-10). 1b. Assess whether PTEN protein loss via IHC predicts for subsequent cancer diagnosis and/or adverse pathologic outcomes in 40 cases of isolated intraductal lesions that did not meet current morphologic criteria for IDC-P (months 4-24). 1c. Validate PTEN IHC assays by correlating with *PTEN* fluorescence *in situ* hybridization (FISH) in 45 IDC-P lesions on tissue microarray (months 6-14).

Task 2: *Profile* the gene expression signature of IDC-P and compare it to that of HGPIN (months 8-36). 2a. Use laser capture microdissection (LCM) to obtain epithelial cells from morphologically-identified IDC-P and PIN occurring with concurrent Gleason 8 tumors and perform DASL and subsequent differential gene expression analysis to establish respective molecular signatures (months 12-36). 2b. Validate the top 3 promising candidate markers for distinguishing IDC-P from HGPIN at the

RNA and protein levels using immunohistochemistry (IHC) and RNA *in situ* hybridization (ISH) on specimens collected in Task 1 (months 24-36).

Task 3: *Integrate* IDC-P into the molecular landscape of invasive carcinoma (months 18-36). 3a. Integrate the expression data for HGPIN and IDC-P into pre-existing, identically-collected datasets for high and low grade invasive tumors using Correspondence at the Top (CAT) plot analysis—supervised by Dr. Luigi Marchionni (months 18-28). 3b. Use the Ampliseq Comprehensive Cancer Panel to compare exomic sequences of 409 oncogenes/tumor suppressor genes in IDC-P with the sequences from the concurrent invasive cancer within each case (n=20 samples total) and confirm a subset of detected mutations using Taqman mutation detection assays (months 18-36). 3c. Use the Nanostring nCounter Cancer Copy Number Assay to compare copy number profile across 86 genes in IDC-P with those in concurrent invasive tumors (n=20 samples total) (months 18-36).

b. What was accomplished under these goals?

Accomplishments for Task 1: For Aim 1, all tasks were completed by August, 2016. The results of tasks 1a and 1b were published (See **Appendix**; Morais CL, Han JS, Gordetsky J, Nagar MS, Anderson AE, Lee S, Hicks JL, Zhou M, Magi-Galluzzi C, Shah RB, Epstein JI, De Marzo AM, Lotan TL. Utility of PTEN and ERG Immunostaining for Distinguishing High Grade PIN and Intraductal Carcinoma of the Prostate on Needle Biopsy. *American Journal of Surgical Pathology*, 2015; 39(2):169-78 PMC: PMC4293206.). In this study, we examined 50 prostate needle biopsies containing invasive tumor with intraductal carcinoma. Of these, 76% (38/50) showed PTEN loss and 58% (29/50) expressed ERG. Of biopsies containing *isolated* intraductal carcinoma, 61% (20/33) showed PTEN loss and 30% (10/33) expressed ERG. Of the borderline intraductal proliferations that did not qualify morphologic criteria as intraductal carcinoma, 52% (11/21) showed PTEN loss and 27% (4/15) expressed ERG. Of the borderline cases with PTEN loss, 64% (7/11) had carcinoma in a subsequent needle biopsy specimen, including 29% (2/7) with Gleason score 6 tumors, 29% (2/7) with a Gleason score 7 tumor, 14% (1/7) with a Gleason score 8 tumor and 29% (2/7) with definitive intraductal carcinoma. The remaining 36% (4/11) of cases with PTEN loss had either PIN or a repeat diagnosis of borderline lesion on subsequent biopsy. Of the PTEN intact cases, 50% (5/10) had a subsequent diagnosis of carcinoma, including 80% (4/5) with Gleason score 6 tumors, 20% (1/5) with Gleason score 7 cancer. Thus, on needle biopsy, PTEN loss is common in morphologically identified intraductal carcinoma yet is very rare in high grade PIN. Borderline intraductal proliferations, especially those with PTEN loss, have a high rate of carcinoma, particularly higher grade (Gleason 7 or higher), on resampling. These results suggest that PTEN and ERG immunostaining may provide a useful ancillary assay to distinguish intraductal carcinoma from high grade PIN in needle biopsies, and we are currently using this assay in our clinical immunohistochemistry lab in this context.

Finally, in an additional publication, we also examined the rate of PTEN loss and ERG rearrangements in PIN occurring in cystoprostatectomy specimens uninvolved by invasive carcinoma, since these specimens do not have possible contamination of PIN-appearing lesions by IDC-P (see **Appendix**, Morais CL Guedes LB, Hicks J, Baras AS, De Marzo AM, Lotan TL. ERG and PTEN Status of Isolated High Grade PIN Occurring in Cystoprostatectomy Specimens Without Invasive Prostatic Adenocarcinoma. *Human Pathology*, 2016; 55:117-25. PMID 7189342). Of 344 cystoprostatectomies,

33% (115/344) contained invasive prostatic adenocarcinoma in the partially submitted prostate (10 blocks/ case on average) and were excluded from the study. Of the remaining cases without sampled cancer, 32% (73/229) showed 133 separate foci of PIN and were immunostained for ERG and PTEN using genetically validated protocols. Of foci of PIN with evaluable staining, 7% (8/107) were positive for ERG. PTEN loss was not seen in any PIN lesion (0/88). Because these isolated PIN foci at cystoprostatectomy are unlikely to represent retrograde spread of invasive tumor, this study confirms PTEN loss essentially never occurs in true neoplastic precursor lesions in the prostate.

Task 1c was also recently completed. Initially, we attempted to correlate the results of PTEN immunohistochemistry (IHC) and PTEN fluorescence in situ hybridization (FISH) in our smaller dataset of IDC-P cases, however on re-sectioning of the block for these experiments, of the tissue microarray, there were too few to do a meaningful analysis of sensitivity, specificity, positive predictive and negative predictive value (less than 30 cases). Thus, we focused on comparing PTEN FISH and our PTEN IHC assay in two large prostate cancer tissue microarray datasets, the Canary Retrospective Tissue Microarray Resource for prostate cancer specimens (n=731 radical prostatectomy specimens) and the Martini clinic cohort (n=4732 radical prostatectomy specimens). In both cohorts, PTEN IHC had high sensitivity and specificity for detection of PTEN gene deletions. For the Canary dataset, the manuscript was recently published (see **Appendix**, Lotan TL, Wei W, Ludkovski O, Morais CL, Guedes LB, Jamaspishvili T, Lopez K, Hawley ST, Feng Z, Fazli L, Hurtado-Coll A, McKenney JK, Simko J, Carroll PR, Gleave M, Lin DW, Nelson PS, Thompson IM, True LD, Brooks JD, Lance R, Troyer D, Squire JA. Analytic Validation of a Clinical-Grade PTEN Immunohistochemistry Assay in Prostate Cancer by Comparison to *PTEN* FISH. *Modern Pathology*, 2016; 29(8):904-14. PMID 27174589.) In this study, intact PTEN immunostaining was 91% specific for absence of *PTEN* gene deletion by FISH, (with 549/602 tumors with 2 copies of *PTEN* showing intact PTEN IHC) and 97% sensitive for homozygous *PTEN* deletion (with detectable PTEN protein loss in 65/67 homozygous tumors). PTEN IHC was 65% sensitive for detection of hemizygous *PTEN* deletion by FISH, with protein loss in 40/62 hemizygous tumors. IHC-guided FISH re-analysis in discordant cases, where IHC showed loss and FISH showed 2 intact copies of *PTEN*, revealed ambiguous IHC loss on re-review in 6% (3/53) cases and failure to analyze the same tumor area in 34% (18/53) cases. Of the remaining discrepant cases, 41% (13/32) revealed hemizygous (n=8) or homozygous (n=5) deletion that was focal in 94% (11/13) cases. Overall, around 10% of the tissue microarray spots contained IDC-P and we did not note any difference in concordance between PTEN IHC and FISH in the IDC-P spots versus the invasive carcinoma spots. Thus, we conclude that PTEN IHC is highly sensitive and specific for PTEN gene deletion.

For the Martini Clinic cohort, the manuscript has been prepared and submission should occur in the next month or so. Overall, similar to the Canary cohort, there was a high concordance between PTEN IHC and FISH (**Table 1**, $p<0.0001$). 93% (3098/3330) of tumors with intact PTEN IHC showed absence of *PTEN* gene deletion and 66% (720/1087) of cases with PTEN protein loss by IHC showed *PTEN* gene deletion by FISH. Similarly, 89% (3098/3465) of tumors with normal *PTEN* by FISH showed intact PTEN IHC and 76% (720/952) of cases with *PTEN* gene deletion by FISH showed PTEN protein loss by IHC. Overall, 84% (447/533) of cases with *PTEN* homozygous gene deletion had PTEN protein loss by IHC. 65% (273/419) of tumors with *PTEN* heterozygous gene deletion showed PTEN protein loss by IHC. Of the discordant cases with PTEN loss by IHC and normal PTEN FISH results, 20% showed heterogeneous PTEN loss. Notably, 20% (74/367) of the discordant cases (loss of PTEN protein expression by IHC and normal *PTEN* by FISH analysis) showed heterogeneous PTEN protein loss in

some, but not all, sampled tumor glands, compared to only 11% (121/1087) of cases with PTEN IHC loss overall which showed heterogeneous PTEN loss. This suggests the possibility that tumor heterogeneity could explain at least some of the discordant results. The negative predictive value for intact PTEN IHC was 93% (3098/3330) for lack of any gene deletion and 97% (3244/3330) for lack of homozygous *PTEN* deletion. The positive predictive value of PTEN IHC loss for presence of any *PTEN* gene deletion (homozygous or heterozygous) was 66% (720/1087) overall, or 70% (673/966) for homogeneous PTEN protein loss and 39% (47/121) for heterogeneous PTEN protein loss.

Accomplishments for Task 2: Approximately 80% completed. We identified 50 cases of isolated PIN and IDC-P, each and selected cases for sectioning in preparation for LCM. These cases were incorporated into a tissue microarray to facilitate validation of the top markers for Task 1. We validated DNA isolation protocols to ensure robust nucleic acid recovery from these small samples, comparing two nucleic isolation methods (Qiagen AllPrep FFPE and QiAmp DNA) and two tumor enrichment methodologies (macrodissection and 0.6 mm tumor block cores). As shown in the prior progress report, these methods are fairly comparable, however RNA is obtained simultaneously from the AllPrep kit, making it the preferable technique. However, we ran into an unexpected challenge with this aim. In the course of these and other studies conducted in our laboratory, and in the laboratory of our collaborator Angelo De Marzo (De Marzo et al, under review), we discovered that RNA from older formalin fixed paraffin embedded (FFPE) specimens (many of the IDC-P cases were more than 10 years old) at Johns Hopkins appeared to be suboptimal in quality and there were some significant differences in RNA from older specimens compared to newer specimens. This meant that we could not use many of the older specimens we initially prepared for DASL, nor could we confidently compare the expression data from the older invasive tumors collected by Dr. Marcionni with the IDC-P cases we were collecting as the FFPE material from these cases was not age-matched. To circumvent this challenge, we are currently prospectively collecting IDC-P and Gleason score 8 invasive cases for analysis.

Since the main goal of task 2b was to discover and develop additional markers for IDC-P, while waiting to collect the prospective cases for RNA expression analyses, we began to develop and validate an additional biomarker for IDC-P, the p53 immunostain. *TP53* missense mutations occur in less than 5% of primary prostate cancers and over 20% of castrate resistant prostate cancer (CRPC). Since *TP53* alterations are highly enriched in lethal prostate cancer, we hypothesized that p53 mutations may be similarly enriched in IDC-P. IHC assays have been used to identify *TP53* mutations in many tumor types, however to our knowledge, no automated, clinical grade assays have been validated for prostate cancer. Thus, we pre-analytically, analytically and clinically validated a robust IHC assay to detect deleterious *TP53* missense mutations in PCa. A p53 IHC assay was developed in a CLIA-accredited laboratory using the BP53-11 monoclonal antibody on the Ventana Benchmark immunostaining system. Benign prostate was entirely negative for p53 protein expression. In cell lines and tissues, p53 protein nuclear accumulation was defined as any p53 nuclear labeling in >10% of tumor cells. For analytic validation, 103 formalin fixed paraffin embedded (FFPE) cell lines from the NCI-60 panel and 47 FFPE PCa tissues (88 primaries adenocarcinomas, 15 CRPC metastases) with known *TP53* mutation status were studied. Mouse xenograft tumors of DU145 and VCaP cell lines both harboring *TP53* missense mutations were subjected to different intervals of delay to tissue fixation and fixation durations to investigate the effects

of pre-analytic variables on immunostaining results. p53 protein nuclear accumulation was 100% sensitive for detection of *TP53* missense mutations in the NCI-60 cell line panel (25/25 missense mutations correctly identified). Lack of p53 nuclear accumulation was 86% (25/29) specific for absence of *TP53* missense mutation. In FFPE prostate tumors, the positive predictive value (PPV) of p53 protein nuclear accumulation for underlying missense mutation was 84% (38/45), while the negative predictive value (NPV) was 97% (56/58). Now that the p53 IHC assay is analytically validated, we are applying it to the biopsy samples from Task 1 as well as the TMA created for Task 2 (see above) to examine the utility of a marker for TP53 mutation in distinguishing IDC-P from PIN, as was done for PTEN above.

Accomplishments for Task 3: Approximately 80% completed. We are awaiting collection of prospective IDC-P cases from Task 2a so that we can correlate somatic genomic alteration status and RNA expression data in these cases. However, we have completed the mutational and copy number analyses on a separate set of invasive cases for comparison as proposed. For Task 3b, we have performed Ampliseq Cancer Panel sequencing, as proposed on more than 50 intermediate/high risk invasive prostate cancer cases for comparison to IDC-P. Exemplary data for the *TP53* gene showing sequencing coverage and correlation with IHC data is shown in **Table 3**. As shown, the coverage is excellent, the DNA requirement is only 150 ng and we have now validated this technique for performance on our IDC-P specimens. For Task 3c, we examined copy number alterations and loss of heterozygosity across 50 cases of invasive carcinoma using Oncoscan FFPE (Affymetrix), a SNP-array based technology using molecular inversion probes. The assay, optimized for FFPE tumor tissue and available in many CLIA labs, uses only 80 ng of DNA input, and provides 50–100 kb copy number resolution in ~900 cancer genes, with 300 kb resolution in all other regions. Compared to the Nanostring assay, this assay is better suited for cases with stromal DNA contamination, as is frequently the case in prostate cancer. We obtained high quality copy number profiles across the majority of cases. Copy number loss at 10q, the PTEN locus, could be correlated with PTEN IHC data, for example. More than 80% of the cases with p53 missense mutation (Task 3b) had one copy allelic loss of chromosome 17p, as expected. A summary of the copy number profiles of these cases demonstrates expected copy number changes in prostate cancer, and validates this technique for copy number calls (**Figure 1**). Though this is the last official reporting period, we will finish the remaining studies of IDC-P proposed (as described above) with institutional funding in the next year and ready them for publication since all techniques are in place for quick assay turnaround once the cases are collected.

c. What opportunities for training and professional development has the project provided?

An excellent opportunity for professional development was afforded by attendance of the IMPACT meeting in Baltimore this year. The data from Task 1 were presented in a podium talk and a poster presentation and it was an excellent opportunity to get feedback on the work and to network with other researchers interested in this area.

d. How were the results disseminated to communities of interest?

In addition to IMPACT presentations described above, two publications have resulted and one more is ready for submission at this time (See **Appendix**).

e. What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report.

4. IMPACT:

a. What was the impact on the development of the principal discipline of the project?

This project has demonstrated, for the first time, that inexpensive and widely available assays used in every pathology laboratory can be used to distinguish two type of pathologic lesions in the prostate that pathologists cannot easily distinguish by eye. Intraductal carcinoma (IDC-P), is a high-risk lesion that is almost invariable associated with high grade invasive cancer, whereas Prostatic Intraepithelial Neoplasia (PIN) looks quite similar to IDC-P, but is not robustly associated with any invasive cancer and is quite common. Using cheap and widely available tests called immunohistochemistry, we have demonstrated that these two lesions can be distinguished on a molecular basis and can add to clinical care of prostate cancer patients. Additional similar assays to this one are currently in the pipeline as developed in this grant. This will change prostate cancer diagnosis by pathologists who can now more easily classify these lesions and relate their relative risks to the clinician.

b. What was the impact on other disciplines?

Nothing to report

c. What was the impact on technology transfer?

Nothing to report

d. What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

a. Changes in approach and reasons for change:

No major changes were undertaken beyond decision to proceed with prospective, rather than retrospective collection of IDC-P specimens based on RNA quality issues describe above.

b. Actual or anticipated problems or delays and action/plans to resolve them:

Prospective collection of IDC-P specimens is proceeding currently as planned.

c. Changes that had significant impact on expenditures:

Nothing to report

d. Significant changes in use or care of human subjects, vertebrate animals, biohazards and/or select agents:

Nothing to report.

6. PRODUCTS:

a. Publications, conference papers and presentations

1) Morais CL, Han JS, Gordetsky J, Nagar MS, Anderson AE, Lee S, Hicks JL, Zhou M, Magi-Galluzzi C, Shah RB, Epstein JI, De Marzo AM, **Lotan TL**. Utility of PTEN and ERG Immunostaining for Distinguishing High Grade PIN and Intraductal Carcinoma of the Prostate on Needle Biopsy. *American Journal of Surgical Pathology*, 2015; 39(2):169-78 PMC: PMC4293206.
2) Lotan TL, Wei W, Ludkovski O, Morais CL, Guedes LB, Jamaspishvili T, Lopez K, Hawley ST, Feng Z, Fazli L, Hurtado-Coll A, McKenney JK, Simko J, Carroll PR, Gleave M, Lin DW, Nelson PS, Thompson IM, True LD, Brooks JD, Lance R, Troyer D, Squire JA. Analytic Validation of a Clinical-Grade PTEN Immunohistochemistry Assay in Prostate Cancer by Comparison to *PTEN* FISH. *Modern Pathology*, 2016; 29(8):904-14. PMID 27174589.
3) Morais CL Guedes LB, Hicks J, Baras AS, De Marzo AM, **Lotan TL**. ERG and PTEN Status of Isolated High Grade PIN Occurring in Cystoprostatectomy Specimens Without Invasive Prostatic Adenocarcinoma. *Human Pathology*, 2016; 55:117-25. PMID 7189342
4) Lotan IMPACT 2016 poster and platform presentation

b. Website(s) or other Internet site(s)

Nothing to report.

c. Technologies or techniques

Nothing to report

d. Inventions, patent applications, and/or licenses

Nothing to report

e. Other Products

Biospecimen collections of IDC-P can be made available through PCBN.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

a. **What individuals have worked on the project?**
   i. Lotan, Tamara –No Change
   ii. Marchionni, Luigi – No Change
   iii. Yegnasubramanian, Srinivasan – No Change
   iv. Benevides Guedes, Liana – No Change
   v. Hicks, Jessica – No Change

b. **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Two new grants have been added (previously pending), no overlap with current award:

Award: PC150699P1
Title: Developing a PTEN-ERG Signature to Improve Molecular Risk Stratification in Prostate Cancer
Effort: 1.2 calendar months or 10%
Supporting Agency: Department of the Army
Name of Procuring Contracting/Grants Officer: Lymor Barnhard
Address of Funding Agency: 820 Chandler Street, Fort Detrick, MD 21702-5014
Performance Period: 10/01/2016-09/30/2019
Level of Funding: $100,000
Principal Investigator: Tamara Lotan (Partnering PI)
Project Goals: The major goal of this project is to develop a gene expression signature for PTEN loss in prostate cancer, stratified by ERG status.
Projects overlap or parallel: No scientific or budgetary overlap.

Award: 1 R01 CA200858-01
Title: Molecular and Cellular Mechanisms of Resistance to mTORC1 Inhibition in the Skin
Effort: 3.6 calendar months or 30%
Supporting Agency: US National Institutes of Health
Name of Procuring Contracting/Grants Officer: Romy Reis
Address of Funding Agency: NIH 616 Executive Boulevard, Suite 7013, MSC 8347, Rockville, MD 20852/
NCI Public Inquiries Office 6116 Executive Boulevard Room 3036A Bethesda, MD 20892-8322
Performance Period: 02/03/2016-1/31/2021
Level of Funding: $200,000
Principal Investigator: Tamara Lotan
Project Goals: The major goal of this project is to elucidate the mechanism by which mTORC1 inhibition up-regulates receptor tyrosine kinase signaling and down-regulates cell-cell adhesion in the skin
Projects overlap or parallel: No scientific or budgetary overlap.

c. **What other organizations were involved as partners?**
Nothing to report

8. FIGURES AND TABLES:

Table 1: Comparison of PTEN IHC and *PTEN* FISH results across all cases with available data from Martini Clinic Cohort

n (%)	**ambiguous IHC**	**PTEN IHC intact**	**PTEN IHC loss heterogeneous**	**PTEN IHC loss homogeneous**
***PTEN* FISH normal**	280 (8%)	3098 (83%)	74 (2%)	293 (8%)
***PTEN* FISH heterozygous deletion**	18 (4%)	146 (33%)	23 (5%)	250 (57%)
***PTEN* FISH homozygous deletion**	17 (3%)	86 (16%)	24 (4%)	423 (77%)

Table 2: Correlation between p53 immunohistochemistry over-expression and presence of *TP53* missense mutation in formalin fixed paraffin embedded prostate tumors

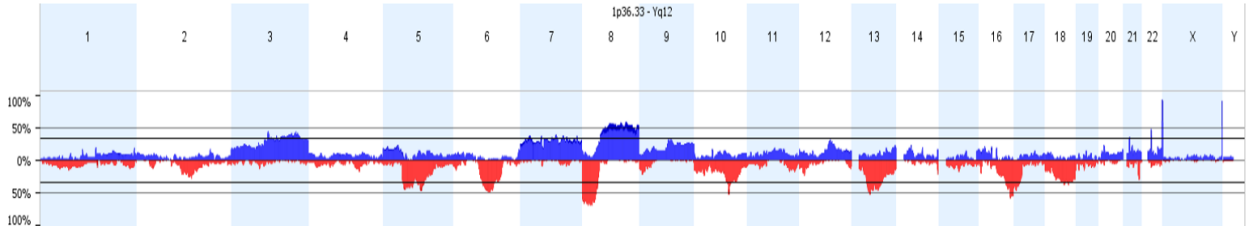
	p53 over-expression	**No p53 over-expression**
***TP53* missense mutation**	38	2
***TP53* WT or deletion**	7	56

Table 3: *TP53* mutation sequencing by Ampliseq Cancer Hotspot Panel. 30 additional genes were also sequenced in this panel.

BlockID	p53 IHC nuclear accumulation	*TP53* Mutation	Allele Frequency	Coverage
22470	1	p.I255delI	0.257	1935
61459	0	p.P190fs*56	0.496	1284
708	0	p.R342*	0.607	1992
2492	0	p.R342*	0.211	1339
33513	0	p.R342*	0.27	1382
34400	1	p.G244D	0.053	1998

34482	1	p.R248L	0.224	1957
60861	1	p.A159V	0.549	1955
17402	1	p.R175H	0.204	588
22953	1	p.R273C	0.249	1179
60868	1	p.E286G	0.483	1109
61467	1	p.G266R	0.347	1111
69189	1	p.F134L	0.501	1967
33768	1	p.R175H	0.626	1710
41598	1	p.S241A	0.45	1951
21861	0	p.R282W	0.609	747

Figure 1: Copy number profiles utilizing Oncoscan across 50 cases of intermediate/high risk invasive prostate cancer at radical prostatectomy. IDC-P copy number profiles will be integrated into this landscape



9. APPENDICES:

Utility of PTEN and ERG Immunostaining for Distinguishing High-grade PIN From Intraductal Carcinoma of the Prostate on Needle Biopsy

Carlos L. Morais, MD, Jeong S. Han, MD,* Jennifer Gordetsky, MD,* Michael S. Nagar, MD,† Ann E. Anderson, MD,† Stephen Lee, MD,* Jessica L. Hicks,* Ming Zhou, MD, PhD,‡ Cristina Magi-Galluzzi, MD, PhD,‡ Rajal B. Shah, MD,§ Jonathan I. Epstein, MD,*‖¶ Angelo M. De Marzo, MD, PhD,*‖¶ and Tamara L. Lotan, MD*‖*

Abstract: Intraductal carcinoma of the prostate and high-grade prostatic intraepithelial neoplasia (PIN) have markedly different implications for patient care but can be difficult to distinguish in needle biopsies. In radical prostatectomies, we demonstrated that PTEN and ERG immunostaining may be helpful to resolve this differential diagnosis. Here, we tested whether these markers are diagnostically useful in the needle biopsy setting. Separate or combined immunostains were applied to biopsies containing morphologically identified intraductal carcinoma, PIN, or borderline intraductal proliferations more concerning than PIN but falling short of morphologic criteria for intraductal carcinoma. Intraductal carcinoma occurring with concurrent invasive tumor showed the highest rate of PTEN loss, with 76% (38/50) lacking PTEN and 58% (29/50) expressing ERG. Of biopsies containing isolated intraductal carcinoma, 61% (20/33) showed PTEN loss and 30% (10/33) expressed ERG. Of the borderline intraductal proliferations, 52% (11/21) showed PTEN loss and 27% (4/15) expressed ERG. Of the borderline cases with PTEN loss, 64% (7/11) had carcinoma in a subsequent needle biopsy specimen, compared with 50% (5/10) of PTEN-intact cases. In contrast, none of the PIN cases showed PTEN loss or ERG expression (0/19). On needle biopsy, PTEN loss is common in morphologically identified intraductal carcinoma yet is very rare in high-grade PIN. Borderline intraductal proliferations, especially those with PTEN loss, have a high rate of carcinoma on resampling. If confirmed in larger prospective studies, these results suggest that PTEN and ERG immunostaining may provide a useful ancillary assay to distinguish intraductal carcinoma from high-grade PIN in this setting.

Key Words: prostatic intraepithelial neoplasia, intraductal carcinoma, prostatic carcinoma, PTEN, ERG

(*Am J Surg Pathol* 2015;39:169–178)

Intraductal carcinoma of the prostate and high-grade prostatic intraepithelial neoplasia (PIN) comprise the 2 main intraepithelial neoplastic lesions occurring in the prostate.[1] When diagnosed as isolated lesions on needle biopsies, these 2 entities have dramatically different implications for patient prognosis and care.[2–4] PIN is widely believed to be a nonobligate precursor lesion of invasive cancer, whereas intraductal carcinoma is a high-grade malignant lesion, likely representing retrograde intraductal/intra-acinar spread of high-grade invasive cancer in most cases.[2,4–11] Accordingly, PIN is frequently an isolated finding, occurring in biopsies without invasive carcinoma and, if not present in at least 2 to 3 separate biopsy cores, is not associated with an increased risk for cancer diagnosis on subsequent biopsies done within the following year.[3] In stark contrast, intraductal carcinoma is associated with underlying high-grade invasive carcinoma in $>90\%$ of cases.[2,11] Whereas many groups do not even recommend rebiopsy for isolated PIN occurring in a single needle core biopsy, most recommend definitive therapy (surgery or radiation) for intraductal carcinoma in a prostate needle core biopsy even without concurrent invasive carcinoma.[2,11] Further, in the presence of concurrent invasive carcinoma, accurate recognition of intraductal carcinoma is also critical as recent studies have established that the presence of this lesion is associated with adverse prognosis after surgery, radiation or neoadjuvant chemotherapy, or hormonal therapy.[12–16]

The distinction of PIN from intraductal carcinoma on needle core biopsy is currently based exclusively on morphologic assessment. Criteria for diagnosis of intraductal carcinoma (and distinction from PIN) have been

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proposed by several groups,[2,6,9] yet even with strict application of these criteria to needle biopsy specimens we and others have encountered a number of cases in which the intraepithelial proliferation shows borderline features, indeterminate between PIN and intraductal carcinoma.[10] Given the critical implications of the diagnosis for patient care, use of an ancillary molecular or immunohistochemical (IHC) test would be helpful in this setting. Recently, using radical prostatectomy specimens, we reported that PTEN protein loss occurs in the majority of morphologically identified intraductal carcinoma cases and was never observed in isolated high-grade PIN.[17] A similar study of borderline intraductal proliferations in radical prostatectomies showed that isolated lesions were entirely negative for ERG, whereas cancer-associated lesions or morphologically identified intraductal carcinoma were highly enriched (75%) for ERG expression.[18] Here, we examined whether immunostaining for PTEN, ERG, and basal cell markers (p63 and high–molecular weight keratin [HMWK]) would be useful to distinguish intraductal carcinoma from high-grade PIN in the more clinically relevant needle biopsy setting.

MATERIALS AND METHODS

Patient and Tissue Selection

This study, including tissue collection and IHC staining, was approved by the authors' Institutional Review Board. Prostate needle biopsy specimens containing intraductal carcinoma with concurrent invasive tumor (n = 50) were collected from the surgical pathology files of the Johns Hopkins Hospitals (JHH), the Cleveland Clinic, and Miraca Life Sciences. Needle biopsies containing isolated intraductal carcinoma (n = 33) without concurrent carcinoma were identified from the consultation files of JHH. All intraductal carcinoma cases were identified applying previously published morphologic criteria[2] and were defined as malignant epithelial cells filling large acini and prostatic ducts, with preservation of basal cells (confirmed by p63 and/or HMWK immunostaining) forming either (1) solid or dense cribriform patterns; or (2) loose cribriform or micropapillary patterns with either marked nuclear atypia (nuclear size >6 times normal or larger) or nonfocal comedonecrosis.

Borderline intraductal proliferations more concerning than high-grade PIN, but falling short of current criteria for intraductal carcinoma, were collected from the consultation files of JHH from 2010 to early 2012 (n = 60). Since 2010, we have diagnosed these cases descriptively as "atypical glands surrounded by basal cells where the differential diagnosis is between high-grade prostatic intraepithelial neoplasia (PIN) and intraductal carcinoma of the prostate" and recommended follow-up biopsies in all cases. The morphologic characteristics of these cases are described in the Results section below. None of these lesions were associated with concurrent infiltrating prostatic adenocarcinoma or a previous known diagnosis of such. Information regarding clinical follow-up was obtained from medical records or from correspondence with outside physicians.

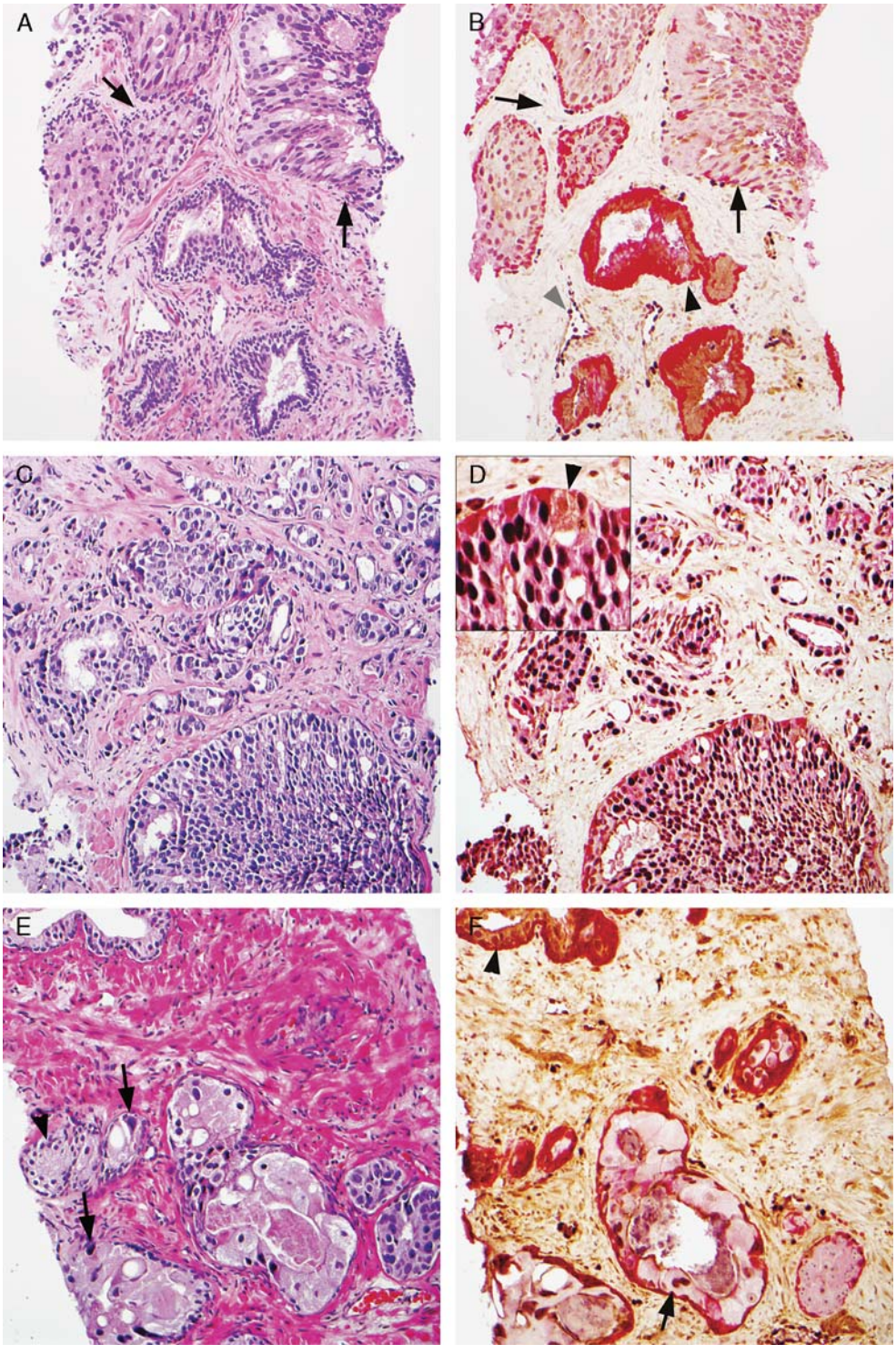
As a control group, we utilized needle biopsies containing high-grade PIN sampled either with (n = 7) or without (n = 12) concurrent carcinoma in additional cores. These cases were identified from the surgical pathology files of JHH from 2010 to 2012. High-grade PIN was defined as a tufted or micropapillary intraepithelial luminal proliferation, identifiable at low power, with nucleoli easily visualized at ×20 magnification.[1] No lesions with cribriform architecture were included in the high-grade PIN group for this study. Of the PIN cases occurring with concurrent carcinoma, 57% (4/7) occurred with Gleason score 3 + 3 = 6 carcinoma and 43% occurred with Gleason 3 + 4 = 7 carcinoma.

Immunohistochemistry

Immunostaining for PTEN, ERG, and basal cell markers was performed using 2 different strategies for cross-validation purposes. On the first subset of cases (30/50 cases of intraductal carcinoma with concurrent invasive adenocarcinoma, 10/33 cases of isolated intraductal carcinoma, and 13/21 cases of borderline intraductal proliferations), we used a 3-color chromogenic quadruple immunostain for PTEN, ERG, p63, and HMWK (34βE12 or CK903) that has been described previously.[17] In this assay, basal cells (p63 and HMWK) are labeled in red (alkaline phosphatase using Vector Red as chromogen), PTEN is labeled in brown (horseradish peroxidase using 3,3′-diaminobenzidine as chromogen), and ERG is labeled in purple (horseradish peroxidase using Vector VIP purple

FIGURE 1. PTEN loss and ERG expression are common in morphologically diagnosed intraductal carcinoma of the prostate on needle biopsy. A, Dense cribriform to solid architecture in an isolated intraductal carcinoma case (arrows). B, Quadruple immunostain for PTEN (brown), ERG (purple), and basal cells (red) on case in (A) demonstrates PTEN loss in intraductal carcinoma (arrows) compared with nearby benign gland (black arrowhead). ERG is expressed in nuclei of intraductal proliferation, although it is less intense than nearby endothelial cells (gray arrowhead). C, Dense cribriform intraductal carcinoma with nearby invasive carcinoma. D, Quadruple immunostain for PTEN (brown), ERG (purple), and basal cells (red) on case in (C) demonstrates PTEN loss and ERG expression in intraductal carcinoma cells (inset) relative to entrapped benign cells (inset, arrowhead). The surrounding invasive carcinoma is concordant with the intraductal carcinoma for these markers. E, Intraductal carcinoma with marked cytologic atypia. Although this case does not show dense cribriform or solid intraductal proliferation, it qualifies as intraductal carcinoma because of the presence of atypical nuclei (arrows) >6× the size of surrounding benign nuclei (arrowhead). F, Quadruple immunostain for PTEN (brown), ERG (purple), and basal cells (red) on case in (E) demonstrates PTEN loss in intraductal carcinoma cells (arrow) relative to nearby benign glands (arrowhead). ERG is also expressed in this case.

as chromogen). To further validate the quadruple immunostain (and in part because the p63 antibody clone 4A4 used in the quadruple immunostain became commercially unavailable during the course of the study), we performed the PTEN, ERG, and HMWK immunostaining analyses individually on adjacent tissue levels on the remainder of the cases, using the same antibody clones as in the quadruple stain, in addition to the previously validated staining protocols.[19,20] Rates of PTEN/ERG staining were nearly identical for each class of lesions using the 2 immunostaining



strategies, further validating the quadruple immunostain's equivalency to the individual stains.

Interpretation of IHC

Cytoplasmic PTEN and nuclear ERG protein were visually scored using a previously validated dichotomous scoring system[19] by a urologic pathologist (T.L.L.). All lesional glands were scored that met morphologic criteria for intraductal carcinoma, borderline intraductal proliferation, or high-grade PIN, based on side-by-side comparisons with a hematoxylin and eosin–stained section. Lesions were scored only if the presence of basal cells could be documented by p63 and/or 34βE12 staining. As previously described,[17,19] cytoplasmic staining for PTEN was classified as negative if the intensity was markedly decreased or entirely negative across >90% of lesional epithelial cells within each gland when compared with the surrounding benign glands and/or stroma, which provide an internal positive control. In a previous study, we found that using this scoring system, PTEN IHC was 100% sensitive and 97.8% specific for *PTEN* genomic loss across a panel of 58 cell lines and between 75% and 86% sensitive for *PTEN* genomic loss in 119 genetically characterized prostate tumor tissues.[19]

Staining for nuclear ERG was assessed in comparison with stromal endothelial cell staining, which provided an internal positive control for ERG in each section. Similarly, adjacent benign glands provided an internal negative control for ERG staining in all cases. Using cutoffs found to be nearly 90% specific for *ERG* gene rearrangement in a prior study,[20] staining for ERG was considered positive if any lesional cells showed nuclear positivity, even those with somewhat weaker staining when compared with surrounding endothelial cells, and negative if no lesional cells were positive.

Statistical Analysis

Fisher exact tests were used to determine the correlation of PTEN and ERG protein expression with one another.

RESULTS

PTEN and ERG Expression in Intraductal Carcinoma and High-grade PIN

Intraductal carcinoma occurring with concurrent invasive tumor showed the highest rate of PTEN protein loss, with 76% (38/50) of cases lacking PTEN protein (Fig. 1, Table 1). In total, 58% (29/50) of these cases expressed ERG. ERG expression was seen in 66% (25/38) of the PTEN loss cases, compared with only 33% (4/12) of the PTEN-intact cases ($P = 0.091$ by the Fisher exact test; Table 2). Overall, 70% (35/50) of cases had concurrent invasive carcinoma present on the same needle core as the intraductal tumor available for analysis. Of these cases, 97% (34/35) showed concordant PTEN and ERG staining between the intraductal and invasive carcinoma. The one discordant case showed PTEN loss in the intraductal component with intact PTEN in the invasive component in the background of negative ERG staining in both components.

TABLE 1. Rate of PTEN Loss and ERG Expression in a Spectrum of Intraepithelial Prostate Proliferations

Intraepithelial Lesion	PTEN Loss (n [%])	ERG Expression (n [%])
Intraductal carcinoma with concurrently sampled invasive carcinoma	38/50 (76)	29/50 (58)
Isolated intraductal carcinoma	20/33 (61)	10/33 (30)
Borderline intraductal proliferations	11/21 (52)	4/15 (27)
PIN with concurrently sampled invasive carcinoma	0/7 (0)	0/7 (0)
Isolated PIN	0/12 (0)	0/12 (0)

Of the needle biopsies containing isolated intraductal carcinoma, 61% (20/33) showed PTEN protein loss and 30% (10/33) expressed ERG. Of the cases with PTEN loss, 50% (10/20) expressed ERG protein, whereas none of the PTEN-intact cases expressed ERG (0/13, $P = 0.0022$ by the Fisher exact test; Fig. 1, Table 3). In contrast, of the high-grade PIN cases occurring with concurrent carcinoma in additional cores, 0% (0/7) showed PTEN loss or ERG expression. Similarly, of the isolated high-grade PIN cases, 0% (0/12) showed PTEN loss or ERG protein expression (Fig. 2, Table 1).

Clinical-pathologic Features of Borderline Intraductal Proliferations Falling Short of Intraductal Carcinoma

We identified 60 cases of borderline intraductal proliferations falling short of current criteria for intraductal carcinoma in our urologic consultation case files from 2010 to early 2012. We limited our search to this period because 2010 was when we first began to formally diagnose these lesions, and we wanted old enough cases to have at least 2 years of clinical follow-up. These cases were characterized by: (1) lumen-spanning proliferation with loose cribriform architecture beyond what would normally be seen in high-grade PIN but lacking significant nuclear pleomorphism or necrosis to qualify for IDC-P (these cases are morphologically similar to those described in our previous radical prostatectomy study as "intraductal cribriform proliferations,"[17]) (Fig. 3A); and/or (2) atypical nuclei with significant pleomorphism but falling short of what is required for a diagnosis of IDC-P (< 6 times larger than adjacent normal epithelial cells) (Fig. 3C); and/or (3) dense cribriform or solid proliferation of atypical cells in incompletely represented large ducts on the edge of core biopsy specimens (Fig. 3E). The majority of cases showed >1 of these features.

Of the 60 cases of borderline lesions, information about subsequent tissue sampling was available in 60% (36/60). Thirty-five of these patients underwent rebiopsy, and 1 underwent an immediate radical prostatectomy despite the fact that he lacked a tissue diagnosis of carcinoma. The remainder of the patients (40%) failed to follow-up with their original urologist or elected to forgo an additional follow-up biopsy despite our recommendation.

TABLE 2. PTEN and ERG Status of Intraductal Carcinoma Sampled With Invasive Carcinoma on Needle Biopsy ($P = 0.091$ by Fisher Exact Test)

	ERG Negative	ERG Positive
PTEN intact	8	4
PTEN loss	13	25

TABLE 3. PTEN and ERG Status of Isolated Intraductal Carcinoma on Needle Biopsy ($P = 0.0022$ by Fisher Exact Test)

	ERG Negative	ERG Positive
PTEN intact	13	0
PTEN loss	10	10

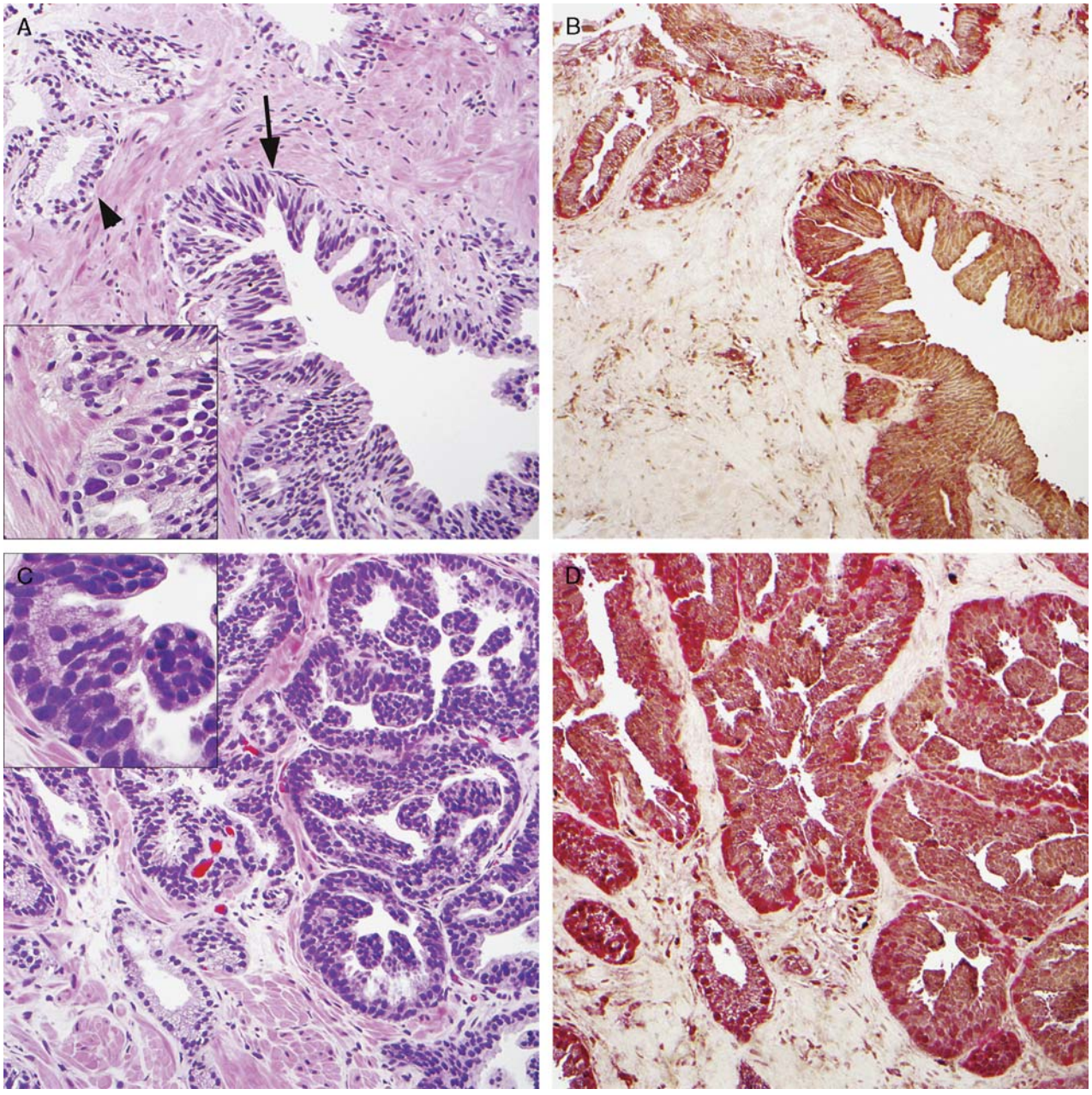


FIGURE 2. PTEN loss and ERG expression are not seen in morphologically diagnosed high-grade PIN on needle biopsy. A, High-grade PIN with tufted architecture (arrow). Nuclear enlargement and nucleoli are apparent at low magnification (arrow) compared with surrounding benign glands (arrowhead). Nucleoli are easily visible (inset). B, Quadruple immunostain for PTEN (brown), ERG (purple), and basal cells (red) on case in (A) demonstrates intact PTEN and absence of ERG staining. C, High-grade PIN with micropapillary architecture. This case contained concurrent invasive adenocarcinoma. D, Quadruple immunostain for PTEN (brown), ERG (purple), and basal cells (red) on case in (C) demonstrates intact PTEN and absence of ERG staining. Nucleoli are easily visible (inset).

Of the 36 patients with additional tissue sampling after a diagnosis of this borderline lesion, the median age was 70 years (range: 56 to 85 y). The number of cores involved by the borderline intraductal proliferation in each case ranged from 1 to 5, with a median of 1. There was a separate focus of atypical glands, suspicious for carcinoma, in 7 of 36 cases (19%). No concurrent invasive carcinoma was diagnosed in any case. The median interval to rebiopsy overall was 4 months, ranging between 0.6 and 3 years. On rebiopsy (or in 1 case, subsequent radical prostatectomy), 50% (18/36) of patients were diagnosed with prostatic carcinoma, with 83% (15/18) showing invasive tumor and 17% (3/18) showing definitive IDC-P. For these patients with a subsequent diagnosis of carcinoma, the median interval to rebiopsy was 5.6 months. For patients with invasive tumor on rebiopsy, 53% (8/15) had a Gleason score of 6, 33% (5/15) had a Gleason score of 7, and 13% (2/15) had a Gleason score of 8. For 2 of the patients with Gleason score 6 carcinoma, the tumor was diagnosed on a second follow-up biopsy, following a rediagnosis of borderline intraductal lesion on the first follow-up biopsy. These were the only 2 patients who had undergone 2 rebiopsies at the time of follow-up. Of the remaining patients, 44% (8/18) showed a borderline intraductal proliferation once again on rebiopsy, 17% (3/18) had a diagnosis of atypical glands, suspicious for prostatic carcinoma, 28% (5/18) showed high-grade PIN on rebiopsy, and 11% (2/18) had a benign diagnosis on rebiopsy.

PTEN and ERG Expression in Borderline Intraductal Proliferations Falling Short of Intraductal Carcinoma

Of the 36 patients who underwent additional tissue sampling after the diagnosis of a borderline lesion, tissue was available for PTEN immunostaining in 58% (21/36) of cases and ERG immunostaining in 42% (15/36). Of these cases, 52% (11/21) showed PTEN protein loss and 27% (4/15) expressed ERG protein (Fig. 3, Table 1). Fifty percent (4/8) of the cases showing PTEN loss expressed ERG compared with 0% (0/7) of the PTEN protein intact cases (Tables 4, $P = 0.0769$ by the Fisher exact test). Of the cases with PTEN loss, 64% (7/11) had carcinoma sampled on a subsequent biopsy, including 29% (2/7) with a Gleason score 6 tumor, 29% (2/7) with a Gleason score 7 tumor, 14% (1/7) with a Gleason score 8 tumor, and 29% (2/7) with definitive intraductal carcinoma. The remaining 36% (4/11) of cases with PTEN loss had either PIN or a repeat diagnosis of borderline lesion on subsequent biopsy. Of the PTEN-intact cases, 50% (5/10) had a subsequent diagnosis of carcinoma, including 80% (4/5) with a Gleason score 6 tumor and 20% (1/5) with Gleason score 7 cancer.

DISCUSSION

Currently, the diagnosis of intraductal carcinoma remains a morphologic one, thus sensitive and specific criteria to accurately distinguish this lesion from common high-grade PIN are essential. As originally defined by McNeal and Yemoto,[6] intraductal carcinoma was characterized by ducts or acini lined by basal cells with an epithelial layer showing cytologic features of moderate-grade to high-grade dysplasia with the additional requirement that luminal extensions of the epithelial lining completely bridged the luminal diameter either as trabeculae or cell masses. Cohen et al[9] proposed 5 major and several minor criteria that built on the original McNeal classification. In contrast to McNeal's criteria, Cohen's criteria included the expansile nature of the lesion, with involved glands more than twice the diameter of normal surrounding peripheral zone glands. Minor criteria included glands that branch at right angles, have smooth contours, and included a dual cell population with more atypical cells at the periphery and maturation toward the center of the lumen. Subsequent criteria put forth by the Epstein group (and utilized in this study) were the most stringent, requiring that the intraluminal proliferation either show a dense cribriform or solid architecture or, if not, have marked cytologic atypia defined on the basis of nuclear size or have comedonecrosis.[2] Application of these criteria to define isolated intraductal carcinoma in prostate needle biopsies was >90% sensitive for detection of underlying invasive carcinoma in subsequent radical prostatectomy specimens.[11]

Given the vastly different clinical implications of the diagnosis of intraductal carcinoma versus the diagnosis of PIN, most morphologic criteria for intraductal carcinoma have emphasized specificity over sensitivity. High-grade PIN most commonly has a tufted or micropapillary architecture with moderate, but not marked, cytologic atypia and nucleoli easily visible at ×20 magnification.[1] In contrast to intraductal carcinoma, solid architecture and comedonecrosis are never seen in PIN; however, the clas-

FIGURE 3. PTEN and ERG expression in borderline intraepithelial proliferations more concerning the PIN, but insufficient for a diagnosis of intraductal carcinoma using current morphologic criteria. A, Borderline proliferation with loose cribriform architecture, unusual for PIN, but insufficient for diagnosis of intraductal carcinoma. B, Quadruple immunostain for PTEN (brown), ERG (purple), and basal cells (red) on case in (A) demonstrates PTEN loss relative to adjacent benign cells (inset shows involved gland from a different area of the core; arrowhead demonstrates a nearby benign gland) and diffuse expression of ERG. C, Borderline proliferation with substantial cytologic atypia (arrow) but lacking sufficient atypia to qualify as intraductal carcinoma. D, Quadruple immunostain for PTEN (brown), ERG (purple), and basal cells (red) on case in (C) demonstrates pagetoid spread of PTEN-negative, ERG-positive cells (arrow). E, Borderline proliferation with dense cribriform architecture, which is highly suspicious for intraductal carcinoma but insufficiently represented at the edge of the needle core. F, Quadruple immunostain for PTEN (brown), ERG (purple), and basal cells (red) on case in (E) demonstrates retention of PTEN and lack of ERG expression in the proliferation.

sification of more loosely cribriform or lumen-spanning intraductal proliferations has been controversial.[2,4,6,7,9–11] Should all lumen-spanning intraductal lesions be considered intraductal carcinoma? Does cribriform PIN exist? Previous studies of so-called "atypical cribriform lesions" using radical prostatectomy specimens have found that the vast majority occur within close proximity to invasive, frequently high-grade, carcinoma.[10] Although a minority occur in isolation from invasive carcinoma and fail to satisfy the criteria for IDC-P, these lesions are relatively rare. Interestingly, although ERG was rearranged in approximately three quarters of atypical cribriform lesions occurring in close proximity to invasive tumors, ERG rearrangement was not seen in isolated atypical cribriform

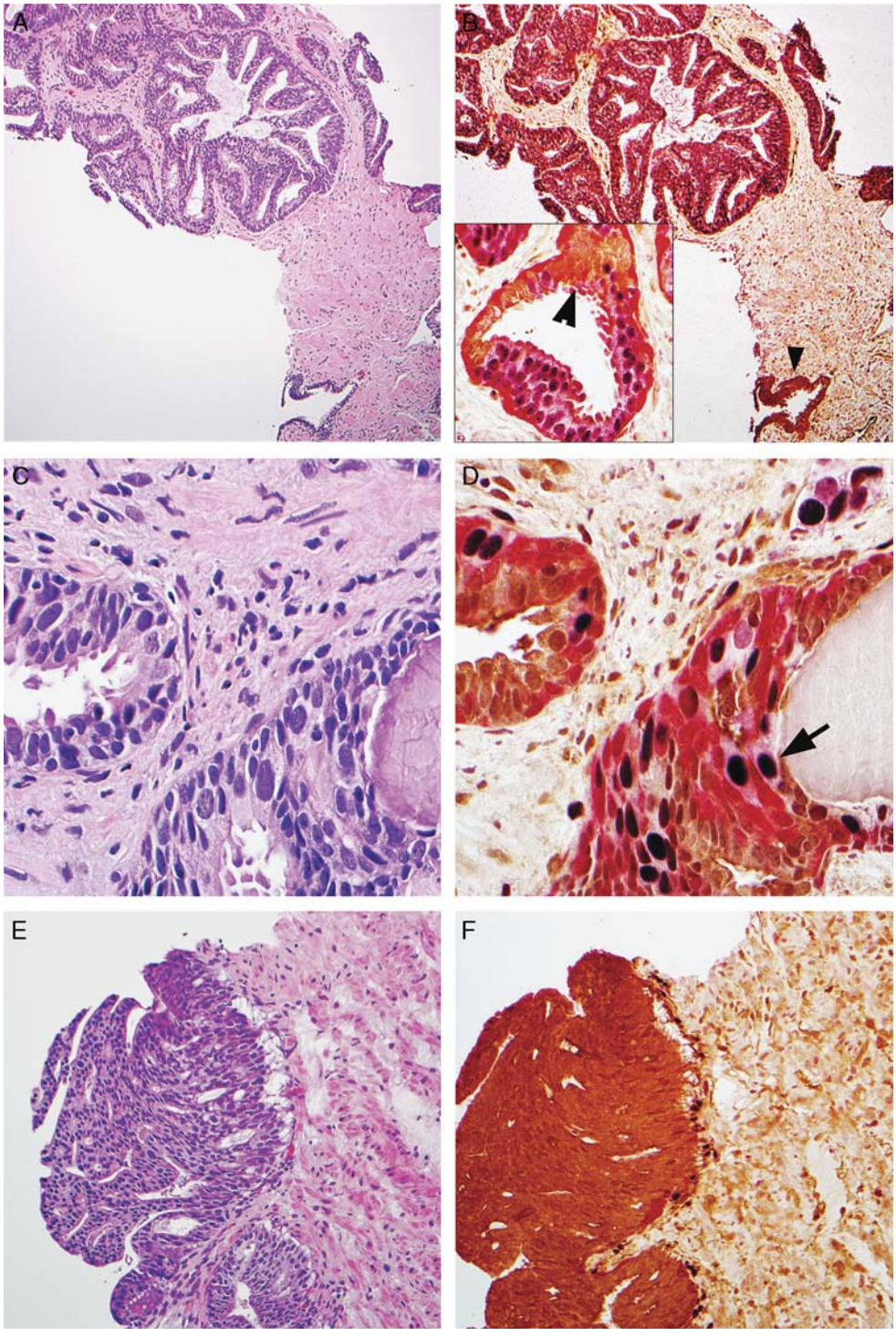


TABLE 4. PTEN and ERG Status of Borderline Intraductal Proliferations Falling Short of Morphologic Diagnosis of Intraductal Carcinoma ($P = 0.0769$ by Fisher Exact Test)

	ERG Negative	ERG Positive
PTEN intact	7	0
PTEN loss	4	4

lesions, suggesting that they may be molecularly distinct and most similar to PIN.[18] Thus, the authors concluded that, although rare true cases of cribriform PIN may exist, these cases are quite uncommon, and, when sampled on needle biopsy, all cribriform intraductal proliferations falling short of intraductal carcinoma should at least undergo a rebiopsy to exclude unsampled carcinoma.

These data and others on radical prostatectomy specimens strongly suggested that ancillary molecular tests may have significant utility for resolving the differential diagnosis of these difficult cribriform lesions.[18,21,22] Because ERG fluorescence in situ hybridization is expensive and time-consuming to perform, and ERG rearrangement can be seen in a subset of conventional PIN cases, in previous work, we focused on the utility of combined PTEN and ERG IHC to distinguish PIN from intraductal carcinoma in radical prostatectomy specimens.[17] In our previous study, we showed that PTEN loss by IHC (which is highly concordant with the presence of an underlying *PTEN* deletion) occurs in >80% of intraductal carcinoma (defined by Epstein criteria) and was not seen in morphologically typical high-grade PIN lesions from grade-matched and stage-matched specimens. The common occurrence of PTEN loss in intraductal carcinoma not only provides a potential marker for this lesion but also suggests a molecular mechanism for the aggressive behavior of tumors associated with intraductal carcinoma. Interestingly, in our previous study, we also examined loose, lumen-spanning cribriform intraepithelial proliferations that fell short of intraductal carcinoma criteria but were adjacent to invasive carcinoma. All of these lesions had loss of PTEN, strongly suggesting that we may be underrecognizing some cases of intraductal carcinoma using current criteria. In this study, ERG was positive in a subset of intraductal carcinomas and frequently concordant with PTEN loss.

Because all previous molecular studies of intraductal lesions have been performed in radical prostatectomy specimens, in which the distinction between intraductal carcinoma and PIN is often straightforward, in the current study we examined these markers in the more clinically relevant setting of prostate needle biopsies. In line with our results from the radical prostatectomy study, we found that over three quarters of morphologically identified intraductal carcinomas occurring with concurrent invasive adenocarcinoma show PTEN protein loss. In isolated intraductal carcinoma sampled without concurrent invasive tumor on needle biopsy, the rate of PTEN loss is similar at 60%. It should be noted that in our previous studies using this same method of PTEN detection by IHC, frequencies of PTEN loss approaching 75% were not seen even in high-grade primary and hormone-naive metastatic prostate carcinomas.[19,23–25] This provides further support for the somewhat unique biological nature of intraductal carcinoma and associated invasive lesions. Strikingly, PIN sampled on needle biopsy with or without concurrent invasive carcinoma did not show PTEN loss in the present study, a finding consistent with our earlier study of PIN in radical prostatectomy specimens.[17] ERG was expressed in 47% (39/83) of intraductal carcinomas overall on biopsy, and its expression was more commonly seen in cases with PTEN loss (60% or 35/58) than those without PTEN loss (16% or 4/25; $P = 0.0002$ by the Fisher exact test), as has been previously reported.[26–29] Interestingly, we did not see ERG expression in the 19 cases of isolated PIN examined in this biopsy study. Prior studies have shown ERG expression in up to 20% of PIN cases; however, it is more commonly seen in PIN adjacent to invasive cancer[30,31] or in isolated PIN diagnosed on needle biopsies from patients with a subsequent diagnosis of invasive cancer.[32]

Taken together, our data suggest that PTEN IHC, either alone or in combination with ERG, may be useful as an ancillary test to distinguish intraductal carcinoma from PIN on prostate needle biopsy. To begin to formally test this hypothesis, we also studied the outcomes of difficult-to-classify borderline intraductal lesions sampled without concurrent carcinoma on needle biopsy. In this category, we included the controversial loose cribriform lesions described above, in addition to intraepithelial lesions with substantial cytologic atypia (but insufficient for a diagnosis of intraductal carcinoma) and lesions only partially represented at the edge of a biopsy core. As a group, these borderline intraductal lesions are analogous to those classified as atypical glands suspicious for carcinoma (ATYP) or atypical small acinar proliferations (ASAP) in that they do not appear to represent an entity in and of themselves, yet their presence in a needle biopsy signifies an increased risk for carcinoma on subsequent biopsies.[1,3,33] In the current series, the risk for carcinoma diagnosis on subsequent biopsy was 50%, slightly higher than that seen after the diagnosis of atypical glands suspicious for carcinoma.[3] Importantly, however, and in contrast to atypical glands suspicious for carcinoma, almost half of borderline intraductal cases with a subsequent diagnosis of invasive carcinoma showed Gleason score of 7 or higher, suggesting that many of these tumors are clinically significant and that a prompt diagnosis is required.

These data strongly suggest that current morphologic criteria for intraductal carcinoma on prostate needle biopsy, although quite specific, may not be optimally sensitive. Thus, we took the first steps to retrospectively examine the utility of PTEN and ERG IHC to predict outcomes in these borderline intraductal lesions. We found that borderline lesions with PTEN loss on needle biopsy had a 64% risk for definitive carcinoma (intraductal or invasive) on subsequent biopsy, a slightly higher risk than seen in the overall population of borderline lesions, and a rate substantially higher than that seen after a diagnosis of a small focus of atypical glands suspicious for carcinoma (ATYP or ASAP).[1,3,33] Although this rate

of carcinoma on rebiopsy was somewhat higher than that seen in the PTEN-intact lesions (64% vs. 50%) or for borderline lesions overall (50%), the current study of borderline lesions has a number of limitations that suggest it is not yet ready for routine clinical use in this context. Perhaps most importantly, it is limited by its modest sample size, as clinical follow-up with available additional tissue for immunostaining was difficult to obtain in our consultation-enriched study population. In addition, our study is limited by the fact that, because of current standards of care, all of the patients were followed up with a needle biopsy, which has limited sensitivity for detection of cancer compared with more thorough examination of a radical prostatectomy specimen. Thus, even if 100% of patients with borderline lesions showing PTEN loss had underlying carcinoma, we would not expect to detect all of these in a single follow-up needle biopsy. The increasing use of magnetic resonance imaging–guided biopsy is rapidly improving the pervasive issue of tumor-undersampling with transrectal ultrasound biopsies. Thus, it is our hope that larger future studies may improve upon our current data and are certainly required before PTEN loss may be used (in combination with morphologic evaluation) to recommend definitive therapy in a borderline intraductal lesion.

Despite these limitations, this study represents the first to use validated molecular markers as an ancillary test to help classify difficult intraepithelial lesions in the prostate with clinical follow-up. Given the clinical significance of distinguishing intraductal carcinoma from high-grade PIN, ancillary molecular tests to help resolve ambiguous cases would be quite valuable to the practicing pathologist. Although these stains (as with all IHC) must always be interpreted in the context of morphology, they may be especially helpful adjunct markers for pathologists who do not see large volumes of urologic material and are less comfortable with the diagnosis of intraductal carcinoma on morphologic grounds alone. Importantly, PTEN loss is only seen in 60% to 70% of classic intraductal carcinoma lesions using current morphologic criteria. This means that if PTEN is intact, this does not rule out a diagnosis of intraductal carcinoma, reducing the negative predictive value of the test, and reinforcing the requirement for morphologic evaluation. In contrast, if PTEN is lost, the positive predictive value is reasonably high, as PTEN loss is rarely if ever seen in morphologically identified PIN.

Further, PTEN loss in an intraepithelial lesion would not only potentially help distinguish it from PIN but, even in a morphologically identifiable case of intraductal carcinoma, would strongly suggest the presence of a concurrent underlying invasive carcinoma with PTEN loss, as these lesions are highly concordant for PTEN status.[17] As we have previously shown that PTEN loss in invasive tumors is strongly associated with higher stage and grade,[19,25] worse outcomes,[19,23,25] and upgrading,[34] this is potentially valuable information to have on a needle biopsy. Although admittedly a small sample size, these data are supported by the current study. More than 70% (5/7) of the patients with borderline intraductal proliferations showing PTEN loss and a subsequent diagnosis of carcinoma had Gleason 7 or higher tumors or intraductal carcinoma (almost invariably associated with Gleason 7 or higher invasive carcinoma[2,11]). In contrast, only 20% (1/5) of the borderline intraductal proliferations with intact PTEN and subsequent carcinoma were diagnosed with Gleason 7 tumor and none with definitive intraductal carcinoma. Given that PTEN loss is only about 60% to 70% sensitive for the detection of intraductal carcinoma on needle biopsy, borderline lesions with intact PTEN not meeting current morphologic criteria for intraductal carcinoma would still need to be followed up with additional biopsies. However, if supported by larger prospective studies, these preliminary results suggest that this simple IHC assay for PTEN may ultimately be useful to help select cases that would benefit from immediate definitive therapy.

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Analytic validation of a clinical-grade PTEN immunohistochemistry assay in prostate cancer by comparison with *PTEN* FISH

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PTEN loss is a promising prognostic and predictive biomarker in prostate cancer. Because it occurs most commonly via *PTEN* gene deletion, we developed a clinical-grade, automated, and inexpensive immunohistochemical assay to detect PTEN loss. We studied the sensitivity and specificity of PTEN immunohistochemistry relative to four-color fluorescence *in situ* hybridization (FISH) for detection of *PTEN* gene deletion in a multi-institutional cohort of 731 primary prostate tumors. Intact PTEN immunostaining was 91% specific for the absence of *PTEN* gene deletion (549/602 tumors with two copies of the *PTEN* gene by FISH showed intact expression of PTEN by immunohistochemistry) and 97% sensitive for the presence of homozygous *PTEN* gene deletion (absent PTEN protein expression by immunohistochemistry in 65/67 tumors with homozygous deletion). PTEN immunohistochemistry was 65% sensitive for the presence of hemizygous *PTEN* gene deletion, with protein loss in 40/62 hemizygous tumors. We reviewed the 53 cases where immunohistochemistry showed PTEN protein loss and FISH showed two intact copies of the *PTEN* gene. On re-review, there was ambiguous immunohistochemistry loss in 6% (3/53) and failure to analyze the same tumor area by both methods in 34% (18/53). Of the remaining discordant cases, 41% (13/32) revealed hemizygous ($n=8$) or homozygous ($n=5$) *PTEN* gene deletion that was focal in most cases (11/13). The remaining 19 cases had two copies of the *PTEN* gene detected by FISH, representing truly discordant cases. Our automated PTEN immunohistochemistry assay is a sensitive method for detection of homozygous *PTEN* gene deletions. Immunohistochemistry screening is particularly useful to identify cases with heterogeneous *PTEN* gene deletion in a subset of tumor glands. Mutations, small insertions, or deletions and/or epigenetic or microRNA-mediated mechanisms may lead to PTEN protein loss in tumors with normal or hemizygous *PTEN* gene copy number.
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PTEN is the most commonly lost tumor suppressor gene in prostate cancer[1–5] and is a promising prognostic biomarker for poor clinical outcomes.[6–18] As the *PTEN* gene is almost always lost by genomic deletion of the entire gene in prostate

tumors, fluorescence *in situ* hybridization (FISH) has traditionally been the gold standard assay to detect *in situ PTEN* loss in tumor tissue. However, the relatively recent availability of reliable rabbit monoclonal antibodies for detection of PTEN protein has enabled the development of highly validated immunohistochemistry protocols to detect PTEN loss in prostate cancer.[9,19] Immunohistochemistry-based detection of PTEN loss in prostate cancer is less expensive and less time-consuming than FISH for the routine screening of prostate tumor specimens, making it easier to adapt to the current pathology workflow for risk assessment in prostate cancer. In addition, because *PTEN* loss is commonly subclonal and heterogeneous in primary prostate tumors,[9,20–22] detection of *PTEN* gene deletion by FISH can be technically challenging in some cases and screening for focal loss may be more easily accomplished by immunohistochemistry. Finally, there is emerging evidence that in addition to genetic deletion, PTEN protein levels may be compromised by mutations in the gene or microRNA- or epigenetic-regulated mechanisms that would not be detectable by FISH.[9,23–25]

We previously optimized and validated a PTEN immunohistochemistry assay for the detection of PTEN loss in prostate cancer specimens,[9] and PTEN loss by this assay correlated with increased risk of biochemical recurrence in a case–control cohort of patients undergoing radical prostatectomy[12] and with risk of progression and metastasis in two high risk surgical cohorts (though the latter was not significant in multivariate analyses).[9,11] Though originally performed manually, we have recently transferred this assay to a clinical-grade automated immunostaining platform that may be run in any CLIA-certified pathology laboratory. Using this assay, we recently reported that PTEN loss is associated with reduced recurrence-free survival in multivariable models in a multi-institutional cohort of surgically treated patients[26] and with higher risk of lethal prostate cancer in a large population-based cohort.[18] *PTEN* gene deletion by FISH has also been recently reported in a subset of the multi-institutional cohort and correlated with recurrence-free survival.[17] Here, to analytically validate our clinical-grade PTEN immunohistochemistry assay, we compared the performance of the automated immunohistochemistry assay with *PTEN* FISH in this cohort, one of the largest multi-institutional cohorts to be studied by both techniques. We demonstrate that our immunohistochemistry assay shows robust sensitivity and specificity for the detection of homozygous *PTEN* gene deletion.

Materials and methods

Subject Selection and Tissue Microarray Design

The Canary Foundation Retrospective Prostate Tissue Microarray Resource has been described in detail elsewhere.[27] Briefly, it is a multicenter, retrospective prostate cancer tissue microarray created as a collaborative effort with radical prostatectomy tissue from six academic medical centers: Stanford University, University of California San Francisco, University of British Columbia, University of Washington (including tissues from University of Washington and a separate cohort from the Fred Hutchinson Cancer Research Center), University of Texas Health Science Center at San Antonio, and Eastern Virginia Medical School. Tumor tissue from 1275 patients was selected for the tissue microarray using a quota sampling plan, from radical prostatectomy specimens collected between 1995 and 2004. A starting date of 1995 was selected to enrich for cases occurring after the implementation of PSA screening. There was no central pathology review in this cohort. The tissue microarray included samples from men with (i) recurrent prostate cancer; (ii) nonrecurrent prostate cancer; and (iii) unknown outcome due to inadequate follow-up time (ie, censoring). Recurrent cases of Gleason score 3+3 = 6 and 3+4 = 7 were relatively over-sampled as well as nonrecurrent cases with Gleason score 4+4 = 8, in order to improve power to detect biomarkers providing prognostic information independent of Gleason score.

Each site built five tissue microarrays, each containing tumor tissue from 42 patients (210 patients from each contributing site). Each tumor was sampled in triplicate, utilizing 1-mm cores and an additional core of histologically benign peripheral zone tissue was included for each patient as a control. Recurrent and nonrecurrent patients were distributed randomly across all tissue microarrays.

Immunohistochemistry Assays

PTEN immunohistochemistry was performed on the CFRPTMR cohort as recently reported.[26] Briefly, the protocol uses the Ventana automated staining platform (Ventana Discovery Ultra, Ventana Medical Systems, Tucson, AZ, USA) and a rabbit anti-human PTEN antibody (Clone D4.3 XP; Cell Signaling Technologies, Danvers, MA, USA). We previously validated a manual version of this assay using the same antibody in genetically characterized cell lines and prostate tumor tissue, showing strong correlation of the immunohistochemistry with *PTEN* gene copy number by two-color FISH and high resolution SNP array analysis[9] and good correlation with four-color FISH in a small cohort of needle biopsy specimens.[28] To prove equivalence between the manual and automated assays, we also examined a test tissue microarray containing 50 prostate cancer cases with known PTEN protein status (including more than 30 with PTEN protein loss) by manual staining and found 100% concordance between the PTEN protein status on the manual and automated platforms.

Immunohistochemistry Scoring

After staining for PTEN, all tissue microarrays were scanned at ×20 magnification (Aperio, Leica Microsystems, Buffalo Grove, IL, USA) and segmented into TMAJ for scoring (http://tmaj.pathology.jhmi.edu/). PTEN protein status was blindly and independently scored by two trained pathologists (TLL and CLM) using a previously validated scoring system (see below). Overall, there was 'very good' agreement between independent reviewers, with 96% agreement over 2783 cores scored by both reviewers ($\kappa = 0.905$; 95% CI = 0.887–0.923).[26]

A tissue core was considered to have PTEN protein loss if the intensity of cytoplasmic and nuclear staining was markedly decreased or entirely negative across >10% of tumor cells compared with surrounding benign glands and/or stroma, which provide internal positive controls for PTEN protein expression.[9] If the tumor core showed PTEN protein expressed in >90% of sampled tumor glands, the tumor was scored as PTEN intact. If PTEN was lost in <100% of the tumor cells sampled in a given core, the core was annotated as showing heterogeneous PTEN loss in some, but not all, cancer glands (focal loss). Alternatively, if the core showed PTEN loss in 100% of sampled tumor glands, the core was annotated as showing homogeneous PTEN loss. Finally, a small percentage of cores were scored as having ambiguous PTEN immunohistochemistry results. This occurred when the intensity of the tumor cell staining was light or absent in the absence of evaluable internal benign glands or stromal staining. The percent of tissue cores with ambiguous scoring for PTEN immunohistochemistry was fairly constant across six of the seven institutions included in the Canary tissue microarray cohort and varied from 0.7 to 5.3%.[26]

For statistical analysis, each patient's tumor sample was scored for the presence or absence of PTEN loss by summarizing the scores of each individual sampled core from that tumor. A patient's tumor was designated as having heterogeneous PTEN loss if at least one tumor core showed heterogeneous PTEN loss, or alternatively, if at least one core showed heterogeneous or homogeneous PTEN loss and at least one core showed PTEN intact in tumor cells. A patient's tumor was scored as showing homogeneous PTEN loss if all sampled tumor cores showed homogeneous PTEN loss. Finally, a patient's tumor was scored as having PTEN intact if all sample tumor cores showed intact PTEN in sampled tumor glands.

Initial Blinded Analysis of PTEN FISH

PTEN FISH was performed as previously described for a subset of this cohort.[17] Briefly, the PTEN DelTECT FISH utilizes a four-color probe combination as described. Probes were supplied by CymoGen Dx LLC (New Windsor, NY, USA) as follows: centromeric copy control probe–CYMO-Pink; *WAPAL*–CYMO-Green; *PTEN*–CYMO-Red; and *FAS*–CYMO-Aqua. We have shown previously that use of the probes bracketing *PTEN* improves the fidelity of assessments of PTEN loss.[29] The two probes *WAPAL* and *FAS* on either side of PTEN provide information about the size of larger deletions and also allow recognition of background artifactual losses of *PTEN* due to histologic sectioning. Artifacts in assessing *PTEN* loss can arise when histologic sectioning cuts away part of the nucleus containing the *PTEN* locus in cells in the section while leaving the centromere in place. The latter is a result of the long distance between the centromere and the *PTEN* locus on chromosome 10.

PTEN FISH analysis was performed entirely independently of PTEN immunohistochemistry, using 5-micron tissue microarray sections stained with DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride) in tumor areas selected by a pathologist who was not involved in PTEN immunohistochemistry scoring (TJ) using an immediately adjacent section stained with hematoxylin and eosin. *PTEN* copy number was evaluated by counting spots for all four probes using SemRock filters appropriate for the excitation and emission spectra of each dye in 50–100 non-overlapping, intact, interphase nuclei per tumor tissue microarray core. For the initial blinded analysis of each case, two tumor-containing cores were scored based on the overall quality of FISH hybridization. In cases where different clonal deletions were present, all three cores were analyzed and more cells were analyzed. Hemizygous (single copy) *PTEN* loss was assigned when >50% of nuclei exhibited either interstitial loss of *PTEN* or concomitant loss of adjacent genes (*PTEN* and *WAPAL* and/or *FAS*). Homozygous deletion was defined by a simultaneous lack of both *PTEN* locus signals in 30% of scored nuclei.

Immunohistochemistry-Guided Re-analysis of Cases with Discrepant Results by Immunohistochemistry and FISH

Fifty-three cases showed PTEN protein loss by immunohistochemistry with two copies of *PTEN* gene present by initial FISH analysis (see Results, below). Two cases showed PTEN protein intact by immunohistochemistry with homozygous *PTEN* deletion by *PTEN* FISH. To analyze the cause of these discrepancies, we re-examined both the immunohistochemistry and FISH data in these cases. A digitally scanned photomicrograph of the most representative core with immunohistochemistry loss was selected to guide FISH re-analysis of the identical core from each case. As the majority (85%) of these discrepant cases showed only focal immunohistochemistry loss in a subset of glands, the FISH re-analyses concentrated on determining the *PTEN* gene copy number within these small areas guided by the immunohistochemistry staining. Because only 50–100 cells from the best two of the

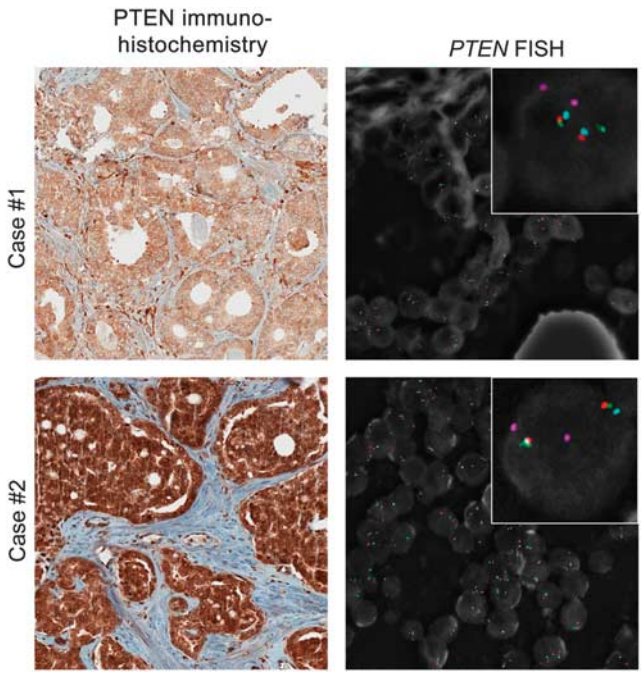


Figure 1 Prostate cancer cases showing intact PTEN protein with two intact PTEN gene alleles. Cases #1 and 2: PTEN immunohistochemistry demonstrates intact PTEN protein (left), while four-color FISH image from adjacent section (right) shows two intact *PTEN* alleles (see enlarged inset–two red signals) with two intact copies flanking genes *WAPAL* (green) and *FAS* (aqua) as well as chromosome 10 centromeres (pink).

three tumor-containing cores were initially analyzed for each case by *PTEN* FISH,[29] this more extensive analysis could include tissue microarray cores and regions of tissue microarray sections that had not been studied by FISH during initial blinded analysis.

Immunohistochemistry and FISH on Standard Tissue Sections

To examine possible effects of tumor heterogeneity on immunohistochemistry and FISH interpretation in the setting of tissue microarray cores, we additionally examined 20 cases of varying PTEN status (enriched for discordance between immunohistochemistry and FISH) by FISH and immunohistochemistry on standard tissue sections. Immunohistochemistry and FISH interpretation of these sections were performed blinded to the results of the tissue microarray analysis and the results of the other methodology.

Results

Data for PTEN FISH and immunohistochemistry in a subset of the CFRPTMR cohort were separately reported previously.[17,26] Briefly, of the 1275 patients with tissue sampled for the tissue microarrays, 86% (1095/1275) had evaluable PTEN status by immunohistochemistry and 14% (180/1275) had missing data (Supplementary Table S1). Of these, 17% (30/180) were missing because of ambiguous immunostaining results and 83% (150/180) had absence of tumor tissue present on the tissue microarray slides. Of the tumors with evaluable staining, 24% (258/1095) showed any PTEN protein loss, with 14% (150/1095) showing heterogeneous PTEN loss (in some but not all sampled tumor glands, best exemplified by case #10 in Figure 4a), and 10% (108/1095) showing homogeneous PTEN loss (in all sampled tumor glands). The remaining 76% (837/1095) of cases had intact PTEN protein by immunohistochemistry in all sampled tumor glands. *PTEN* FISH results were evaluable in 64% of the cases sampled on the tissue microarray (810/1275). Of the evaluable cases, *PTEN* FISH showed any *PTEN* deletion in 18% of cases, with 9% (70/810) of cases showing hemizygous deletion and 9% (75/810) of cases showing homozygous *PTEN* deletion. The remaining 82% (665/810) of cases showed two intact *PTEN* alleles.

PTEN immunohistochemistry results were available on 90% of cases with interpretable *PTEN* FISH results (731/810). The rates of *PTEN* gene and PTEN protein loss were quite similar in the subset with both FISH and immunohistochemistry results compared with the entire evaluable cohort for each assay reported separately. Overall, 22% (158/731) of cases with interpretable immunohistochemistry and FISH results showed PTEN protein loss, with 13% (96/731) showing heterogeneous loss and 8% (62/731) showing homogeneous loss. Similarly, 17% (129/731) of cases with interpretable immunohistochemistry and FISH results showed *PTEN* gene deletion (8% hemizygous and 9% homozygous).

Overall, intact PTEN immunohistochemistry was 91% specific for lack of underlying *PTEN* gene deletion. Of cases with two copies of the *PTEN* gene by FISH analysis, 549/602 showed intact PTEN protein (Figure 1, Tables 1 and 2). Notably, 85% (45/53) of the discrepant cases (loss of PTEN protein expression by immunohistochemistry and two copies of *PTEN* gene by FISH analysis) showed heterogeneous PTEN protein loss in some, but not all, sampled tumor glands, suggesting the possibility that a small area with *PTEN* deletion may have been missed in the initial FISH analysis (see below). PTEN immunohistochemistry loss was 65% sensitive for the detection of underlying hemizygous *PTEN* gene deletion because 40/62 of cases with hemizygous *PTEN* gene deletion by FISH showed PTEN protein loss by immunohistochemistry (Figure 2). Of these cases, 65% (26/40) showed heterogeneous PTEN loss in some but not all sampled tumor glands. PTEN immunohistochemistry loss was 97% sensitive for homozygous *PTEN* gene deletion. Of cases with homozygous gene deletion by FISH, 65/67 showed PTEN protein loss by immunohistochemistry (Figure 3). Only 37% (25/67) of the cases with homozygous *PTEN* gene deletion and PTEN protein loss had heterogeneous loss of PTEN protein by immunohistochemistry. The fraction of tumors with

Table 1 Summary of PTEN immunohistochemistry by PTEN FISH status

	PTEN FISH					
	Intact		*Hemi-deletion*		*Homo-deletion*	
	N	%	N	%	N	%
PTEN immunohistochemistry						
Intact	549	91	22	35	2	3
Heterogeneous loss	45	7	26	42	25	37
Homogeneous loss	8	1	14	23	40	60

Table 2 Performance metrics for PTEN immunohistochemistry compared to gold standard *PTEN* FISH

	%	n
Specificity	91	549/602
Sensitivity for homozygous deletion	97	65/57
Sensitivity for hemizygous deletion	65	40/62
Positive predictive value	66	105/158
Negative predictive value	96	549/573

underlying homozygous *PTEN* gene deletion differed by the extent of PTEN protein loss observed: 26% (25/96) tumors with heterogeneous PTEN protein loss had an underlying homozygous *PTEN* deletion compared with 64% (40/62) of tumors with homogeneous PTEN protein loss ($P < 0.0001$ by Fisher's exact test).

The negative predictive value for intact PTEN immunohistochemistry was 96% (549/573) for lack of any gene deletion and 99.6% (571/573) for lack of homozygous *PTEN* deletion (Table 2). The positive predictive value of PTEN immunohistochemistry loss for presence of any *PTEN* gene deletion (homozygous or hemizygous) was 66% (105/158) overall, or 53% (51/96) for heterogeneous PTEN protein loss and 87% (54/62) for homogeneous PTEN protein loss (Table 2).

Next, we re-examined cases where there was a discrepancy between the PTEN immunohistochemistry and FISH. Overall, 53 cases with PTEN protein loss had two intact copies of *PTEN* by FISH, of which 85% (45/53) showed heterogeneous PTEN protein loss. Because only 50–100 tumor cells from two of the three tumor cores from each cases were initially evaluated by FISH, it is possible that focal tumor areas with *PTEN* gene deletion by FISH were missed or not analyzed in this blinded analysis. To examine this and other possible explanations for the immunohistochemistry-FISH discrepancy, each of these 53 discordant cases were re-reviewed for immunohistochemistry and FISH staining. Immunohistochemistry-guided FISH re-analysis in these cases revealed borderline immunohistochemistry loss in 6% (3/53) cases (Figure 4a, Case #10) and failure to analyze the identical tumor core or area by both immunohistochemistry and FISH in

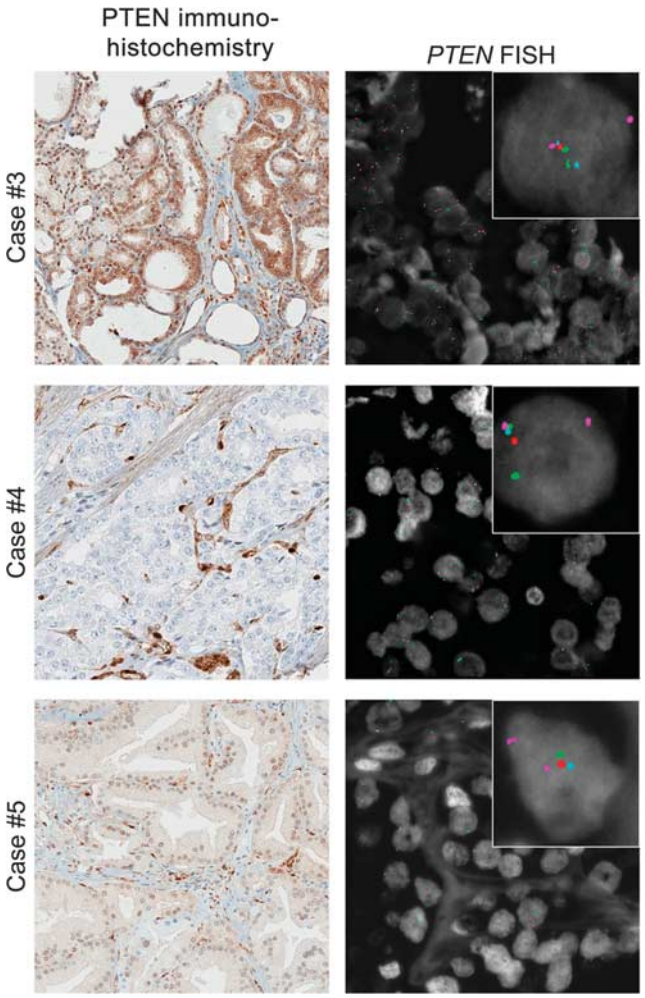


Figure 2 Prostate cancer cases showing variable PTEN protein expression with hemizygous *PTEN* gene deletion. Case #3: PTEN immunohistochemistry demonstrates intact PTEN protein (left), with four-color FISH image from an adjacent section showing a hemizygous *PTEN* deletion with loss of one *PTEN* gene (see enlarged inset-one red signal). As both centromeres (pink) and the *WAPAL* (green) and *FAS* (aqua) probes that flank either side of *PTEN* are retained, it is likely that this hemizygous deletion is interstitial and restricted to the *PTEN* region. Case #4: PTEN immunohistochemistry image shows homogeneous loss of PTEN protein (left) while FISH image from an adjacent section (right) shows a hemizygous *PTEN* deletion (see enlarged inset–one red signal). Concurrent hemizygous deletion of the adjacent *FAS* gene probe (one aqua signal missing) but retention of two copies of the centromere and *WAPAL* gene probes indicates the deletion includes both the *PTEN* and *FAS* genes. Case #5: PTEN immunohistochemistry image shows somewhat light, but intact immunostaining for PTEN protein (left) while the FISH image from an adjacent section (right) shows a hemizygous *PTEN* deletion (see enlarged inset–one red signal). Because there was concurrent loss of the *WAPAL, PTEN,* and *FAS* gene probes (green, red, and aqua, respectively), but retention of both centromeres (pink), this hemizygous deletion extends outside the *PTEN* region in both directions.

34% (18/53) cases. Of the remaining discrepant cases where the immunohistochemistry result was convincing and the identical tumor area was analyzed by both methods, 41% (13/32) revealed hemizygous ($n = 8$, Figure 4a, Case #11) or homozygous ($n = 5$,

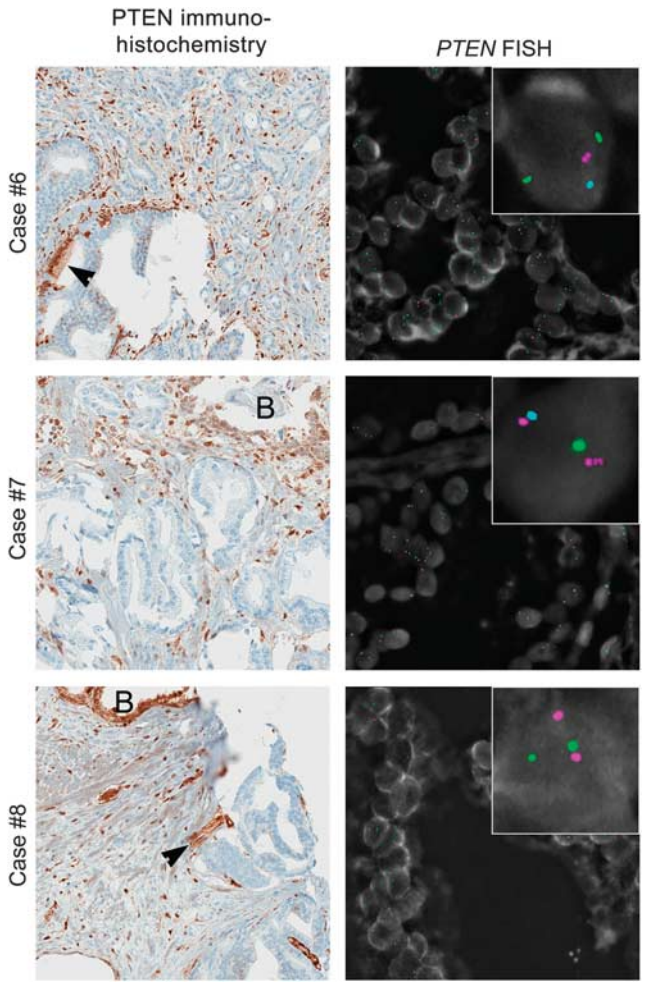
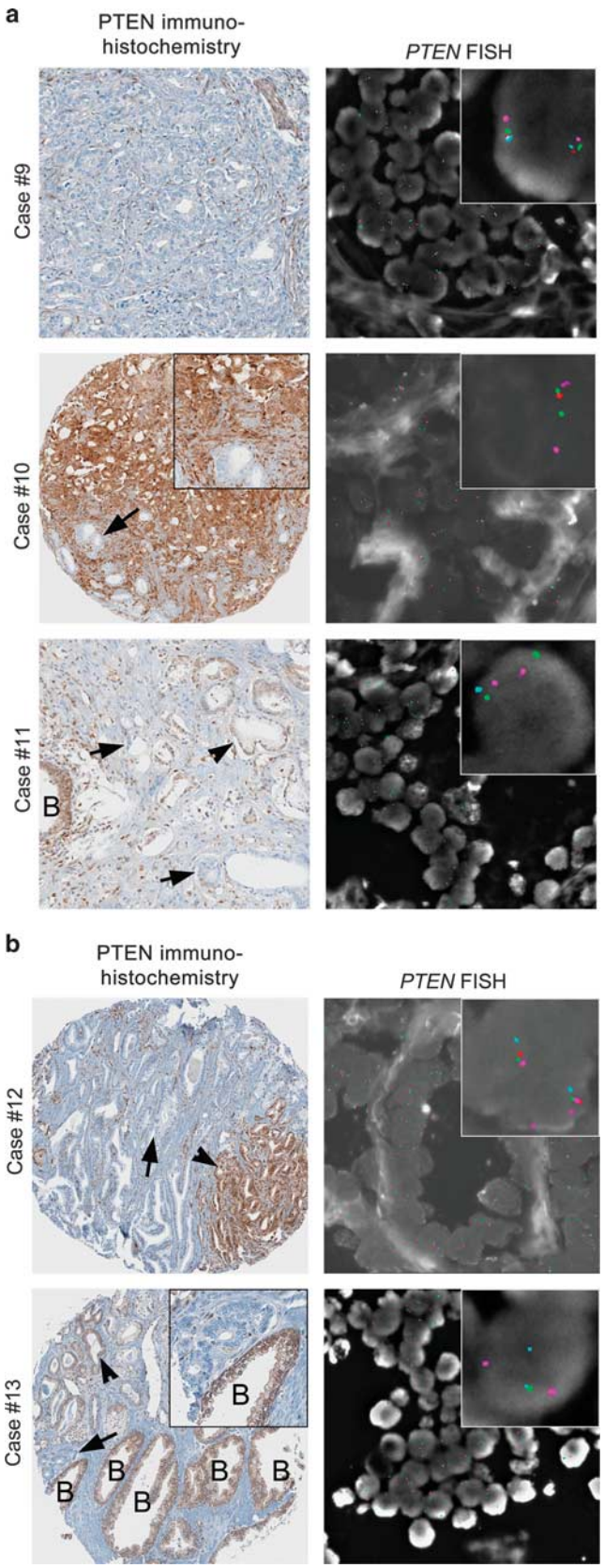


Figure 3 Prostate cancer cases showing absence of PTEN protein expression with homozygous *PTEN* gene deletion. Case #6: PTEN immunohistochemistry image (left) shows loss of PTEN protein in tumor glands. Intraductal spread of tumor is present in this case and retention of PTEN protein is seen in benign basal and luminal cells of duct containing tumor (arrowhead). Four-color FISH image from an adjacent section (right) shows a homozygous deletion with loss of both *PTEN* genes (see enlarged inset–no red signals). The retention of the centromeres (pink) and both *WAPAL* genes (green), but the presence of only one copy of the *FAS* gene (aqua) indicates that one of the deletions involved both the *PTEN* and *FAS* genes. Case #7: PTEN immunohistochemistry image (left) shows loss of PTEN protein in tumor glands, with retention in entrapped benign gland (B). FISH image from an adjacent section (right) shows a homozygous *PTEN* deletion (see enlarged inset–no red signals). The retention of the centromeres (pink) but concurrent loss of one *WAPAL* (green) and one *FAS* gene (blue) indicates the deletions extend outside the *PTEN* region. Case #8: PTEN immunohistochemistry image (left) shows loss of PTEN protein in tumor glands, with retention in adjacent benign gland (B) and nearby endothelial cells (arrowhead). (FISH image from an adjacent section (right) shows a homozygous *PTEN* deletion (see enlarged inset–no red signals). The retention of the centromeres and both the *WAPAL* genes (green), but the concurrent loss of both *FAS* (blue) and *PTEN* (red), indicates that both copies of chromosome 10 have deletions involving these genes.

Figure 4a, Case #12) deletion that was focal in 94% (11/13) cases and thus likely missed on initial FISH analysis. The remaining 59% (19/32) of these cases showed two copies of *PTEN*, thus representing truly discordant cases. One explanation for these cases is the presence of a small deletion and/or mutation undetectable by FISH at one or both *PTEN* alleles. Another possibility is that even though the same core was evaluated by both methods in these cases, there may be heterogeneity within the core such that different levels of the core sampled on the FISH and immunohistochemistry slide may have been truly heterogeneous (Figure 4b, Case # 13). Of the two discrepant cases with homozygous *PTEN* deletion and intact PTEN protein, different tumor areas were analyzed in one case. In the other case, a minute focus of tumor with PTEN loss by immunohistochemistry that was initially missed was observed on re-examination (Figure 4b, Case # 14).

Finally, to further assess the effects of tumor heterogeneity on PTEN immunohistochemistry and FISH results and to determine whether this might account for discordance in some cases, we blindly studied a subset of 20 cases from the tissue microarray using standard tissue sections and compared results of immunohistochemistry and FISH on standard sections with one another and with those obtained for the tissue microarray cores of the same cases (Table 3). Cases chosen for this analysis were relatively enriched for discordance between tissue microarray-based immunohistochemistry and FISH results. In cases where the immunohistochemistry and FISH were concordant on the tissue microarray cores, results were generally highly concordant using standard sections as well. For example, in three cases where there was heterogeneous PTEN loss by immunohistochemistry and homozygous *PTEN* loss by FISH in the tissue microarray cores, two of these tumors had clonal homozygous *PTEN* deletions, and the third tumor had a region with homozygous loss surrounded by a larger area with *PTEN* hemizygous loss. Similarly, in four cases that were PTEN intact by both immunohistochemistry and FISH on tissue microarray cores, three showed *PTEN* intact by FISH on standard sections (the fourth case failed to hybridize) and three showed PTEN intact by immunohistochemistry on standard sections (the fourth case showed focal PTEN loss). In some cases where there was discordance between the immunohistochemistry and FISH results on tissue microarray cores, more detailed analysis of standard sections suggested that tumor heterogeneity may be the underlying cause. Of eight cases with heterogeneous PTEN loss by immunohistochemistry and intact *PTEN* by FISH on tissue microarray, four showed either hemizygous or homozygous *PTEN* loss by FISH on standard sections. Another case with homogeneous PTEN loss by immunohistochemistry and intact *PTEN* by FISH on tissue microarray revealed hemizygous *PTEN* loss by FISH on analysis of standard sections. Overall, these results support the possibility that underlying tumor heterogeneity is one potential cause of PTEN immunohistochemistry-FISH discordance. Despite this, tissue microarray-based



evaluation of tumor PTEN status appears to be highly concordant with standard section analysis in most cases.

Discussion

There is an increasing need for validated prognostic and predictive biomarkers in prostate cancer at both ends of the clinical spectrum. Developing prognostic biomarkers to help select patients who are appropriate for active surveillance as well as predictive biomarkers to guide the application of targeted therapy in metastatic disease remain major areas of unmet clinical need. PTEN has long been a promising marker in both regards, however, until relatively recently, the lack of well-validated antibodies to detect PTEN loss has made it challenging to incorporate into routine pathologic risk assessment

Figure 4 (**a**) Prostate cancer cases with discordant PTEN immunohistochemistry and FISH results on initial review. Case #9: PTEN immunohistochemistry demonstrates very weak cytoplasmic immunostaining with loss of nuclear immunostaining and thus was called negative on initial review, though in retrospect, it may be better classified as ambiguous because of weak staining and absence of benign glands for comparison (left). Four-color FISH image from an adjacent section that is representative of all examined cores in this tissue microarray (right) indicates that the *PTEN* gene does not have a detectable deletion by FISH. The enlarged inset shows that the centromeres, *WAPAL, PTEN*, and *FAS* gene probes are each present as two copies. Case #10: PTEN immunohistochemistry image (left) shows heterogeneous PTEN loss in some tumor glands (arrow), but PTEN protein is expressed by the majority of other tumor glands in this core. FISH image from an adjacent section (right) was initially read as PTEN intact, but shows a focal area with hemizygous *PTEN* deletion recognized on re-examination guided by immunohistochemistry. The enlarged inset shows that there is only one copy of the red *PTEN* gene probe (one red signal) and loss of both aqua *FAS* gene probes. Case #11: PTEN immunohistochemistry image (left) demonstrates heterogeneous PTEN loss in some tumor glands (arrows) but not in others (arrowheads). FISH image from an adjacent section (right) shows the small area of the section that had a homozygous *PTEN* deletion on re-examination. The enlarged inset shows that there are no copies of the red *PTEN* gene probe and one copy of the aqua *FAS* gene probe, but retention of the adjacent *WAPAL* and centromere probes. (**b**) Prostate cancer cases with discordant PTEN immunohistochemistry and FISH results on initial review. Case #12: PTEN immunohistochemistry image (left) shows heterogeneous loss of PTEN protein in some tumor glands (arrow) but not in others (arrowhead). A FISH image from an adjacent section that is representative of all examined cores in this tissue microarray (right) indicates that the *PTEN* gene does not have a detectable deletion by FISH. The enlarged inset shows that the centromeres, *WAPAL, PTEN,* and *FAS* gene probes are each present as two copies. The heterogeneous loss in this case may have resulted in different tumor areas sampled in slides for immunohistochemistry and that for FISH. Case #13: PTEN immunohistochemistry image (left) shows predominantly intact/light immunostaining in tumor glands (arrowhead) and benign glands (**b**) with a very focal area of tumor with PTEN loss identified on re-review after FISH analysis (arrowhead, inset). FISH analysis of an adjacent section to the immunohistochemistry indicates a homozygous *PTEN* deletion. The enlarged inset shows that there are no copies of the red *PTEN* gene probe and loss of one green *WAPAL* gene probe but retention of both the *FAS* and the centromere probes.

Table 3 Comparison of PTEN immunohistochemistry and FISH results on tissue microarray cores and standard tissue section slides

Case	*Tissue microarray PTEN immunohistochemistry*	*Standard slide PTEN immunohistochemistry*	*Tissue microarray PTEN FISH*	*Standard slide PTEN FISH*
1	Intact	Intact	Intact	Intact
2	Intact	Heterogeneous loss	Intact	Intact
3	Intact	Intact	Intact	Intact
4	Intact	Intact	Intact	Failure
5	Heterogeneous loss	Heterogeneous loss	Homo-deletion	Hemi-deletion and homo-deletion
6	Heterogeneous loss	Heterogeneous loss	Homo-deletion	Homo-deletion
7	Heterogeneous loss	Heterogeneous loss	Homo-deletion	Homo-deletion
8	Heterogeneous loss	Heterogeneous loss	Intact	Intact
9	Heterogeneous loss	Heterogeneous loss	Intact	Intact
10	Heterogeneous loss	Intact	Intact	Intact
11	Heterogeneous loss	Heterogeneous loss	Intact	Intact
12	Heterogeneous loss	Intact	Intact	Hemi-deletion
13	Heterogeneous loss	Heterogeneous loss	Intact	Homo
14	Heterogeneous loss	Heterogeneous loss	Intact	Homo
15	Heterogeneous loss	Heterogeneous loss	Intact	Hemi-deletion of *WAPAL*
16	Homogeneous loss	Homogeneous loss	Hemi-deletion	Hemi-deletion
17	Homogeneous loss	Heterogeneous loss	Intact	Hemi-deletion
18	ambiguous	Intact	Intact	Intact
19	Heterogeneous loss	Heterogeneous loss	Core missing	Intact
20	Heterogeneous loss	Heterogeneous loss	Core missing	Intact

protocols or clinical trials of PI3K-targeted agents in prostate cancer. Owing to this difficulty, FISH has historically been used to assess whether PTEN is an effective prognostic biomarker by testing the association of *PTEN* gene deletion with prostate cancer progression. The results from these studies have consistently shown that *PTEN* gene deletion is associated with increased Gleason grade and stage in prostate cancer.[6,8,10,17,30,31] In addition, *PTEN* gene deletion is associated with prostate cancer progression and death in multivariable models.[6–16] Though many of these previous studies have used two-color FISH, there is increasing evidence that four-color probes are better suited to distinguish true gene deletions from sectioning artifacts in interphase FISH studies (Yoshimoto *et al,* in preparation). Accordingly, our group recently demonstrated that homozygous PTEN deletion by four-color FISH is associated with decreased recurrence-free survival in a subset of the prostate tumor cohort examined in the current study.[17]

Despite these compelling data, *PTEN* FISH has not been widely implemented in clinical prostate cancer risk stratification protocols to date for a number of reasons. First, FISH to detect gene deletions is technically challenging, requiring careful probe design[29] and rigorous cutoffs to ensure that sectioning artifacts do not result in false calls of deletion. Detection of hemizygous deletions can be particularly challenging when nuclei are overlapping or have been distorted during preparation. Depending on tissue quality and fixation, there may also be difficulties with optimizing protease digestion such that as many as 30–40% of cases cannot be evaluated on the first attempt when using tissue microarrays, though this may be less of an issue for biopsies.[17] In large part because it is so technically challenging, FISH is relatively expensive compared with immunohistochemistry, and it has been harder to integrate the daily workflow of pathology laboratories as a reflexive test. Finally, though *PTEN* is most commonly lost via larger genomic deletions in prostate cancer, as many as 10–20% of cases may have mutations, small insertions, or deletions that are not detectable by FISH, in addition to potential epigenetic and miRNA-mediated mechanisms of PTEN loss.[1–5,32] To address these challenges, several groups have developed immunohistochemistry assays to query PTEN status in tissue.[9,19,33] Although a number of such assays have been published, for the most part, these assays have largely been compared with two-color FISH in only small-scale studies with around 100 tumors each.[23,24,34,35] In the only large studies to compare immunohistochemistry and FISH, there was only weak ($\kappa = 0.5$)[14] or no significant correlation[13] between the assays, suggesting a failure of the immunohistochemistry and/or FISH assay to analytically validate.

We used a commercially available rabbit monoclonal antibody to develop an immunohistochemistry assay to assess PTEN protein loss in prostate cancer and showed that this assay is reasonably sensitive for the detection of *PTEN* gene deletion by two-color FISH or high density SNP array in prostate cancer samples and shows minimal inter-observer variability in interpretation.[9] Similarly, the assay performed well *vs* four-color FISH in a small cohort of needle biopsy specimens.[28] Using this assay, our group previously demonstrated that PTEN protein loss is associated with an increased risk of recurrence and progression in surgically treated cohorts of prostate cancer patients.[11,12]

To facilitate clinical use of the assay, we adapted it to the automated Ventana staining platform with

clinical-grade reagents suitable for *in vitro* diagnostic use. This assay was clinically validated in a recent study showing that PTEN loss is associated with increased risk of lethal prostate cancer in a large population-based cohort in multivariable models.[18] Despite a 4-category scoring system, the assay has shown high inter-observer reproducibility in a number of cohorts (including the current one), with κ values exceeding 0.9.[18,26] In the current study, we analytically validated this automated assay by comparing it to four-color *PTEN* FISH across a large multi-institutional cohort of prostate cancer patients. Remarkably, we found that the automated immunohistochemistry assay was 91% specific for two intact copies of the *PTEN* gene and 97% sensitive for homozygous *PTEN* gene deletions. This is by far the highest sensitivity and specificity reported for a PTEN immunohistochemistry assay relative to FISH. This improved sensitivity and specificity is in part because of the improved specificity of the automated immunohistochemistry assay *vs* the manual assay and also because of the improved four-color FISH assay, which uses two *PTEN* gene-flanking probes, in addition to a centromeric control and a *PTEN* probe to detect *PTEN* gene deletions. Surprisingly, the immunohistochemistry assay was also 65% sensitive for detection of hemizygous *PTEN* gene deletion, suggesting that there is complete protein loss in a large fraction, perhaps even a majority, of apparently hemizygous cases. This is most likely due to truncating mutations (nonsense, frameshift, and splice site mutations) or epigenetic modifications at the second allele that are undetectable by FISH yet lead to protein loss.[1,3,5,36] Interestingly, though the prevalence of such mutations in *PTEN* is below 5% in most prostate tumors, many of these mutations are truncating alterations occurring in cases with hemizygous deletions that would lead to protein loss detectable by immunohistochemistry.[1–5]

In addition to the potential increased sensitivity of immunohistochemistry *vs* FISH for detecting combinations of events including copy loss, point mutations, small insertions and deletions, and epigenetic modifications leading to *PTEN* inactivation, immunohistochemistry is also very useful for screening for areas of focal PTEN loss. By necessity, *PTEN* FISH is analyzed at high magnification, examining 50–100 nuclei, which may miss small areas of loss within the sampled tumor. In contrast, immunohistochemistry can be easily screened at low magnification and still afford a nearly cell-by-cell resolution image of PTEN expression. In the current study, in over 40% of cases where PTEN immunohistochemistry detected loss and *PTEN* FISH was initially read as two copies in the identical tumor core, rescreening the FISH guided by areas of immunohistochemistry loss resulted in detection of small areas with *PTEN* deletion, initially missed or beneath the cutoff for the FISH scoring. This result, in addition to the high negative predictive value of intact immunohistochemistry for lack of deletion strongly suggests that immunohistochemistry screening for PTEN loss is likely to be an efficient and cost-effective strategy to ascertain PTEN status in tissue sections.

Akin to HER2 assessment in breast, it is ultimately likely that the best protocol will be to perform reflexive FISH on a subset of prostate tumors after initial immunohistochemistry screening. Clearly, in cases with ambiguous immunohistochemistry results ($<5\%$), FISH will have an important role. However, there may also be a role for FISH in cases with heterogeneous loss of PTEN by immunohistochemistry. As in previous cohorts,[12] in the current cohort, we found that homogeneous PTEN immunohistochemistry loss was more strongly associated with decreased recurrence-free survival compared with heterogeneous PTEN protein loss in both univariate and multivariate analyses.[26] The explanation for why focal PTEN loss is a less potent prognostic indicator than homogeneous loss remains unclear. Homogeneous PTEN loss may be a surrogate indicator for expansion of a single, dominant clone of tumor cells. Alternatively, perhaps loss of PTEN in a larger number of cells increases risk of tumor progression for stochastic reasons. Finally, this result may also be related to the higher prevalence of homozygous *PTEN* deletion among the cases with homogeneous immunohistochemistry loss, compared with the cases with heterogeneous immunohistochemistry loss (64 *vs* 26%; $P<0.0001$ by Fisher's exact test). Indeed, in the subset of the current cohort where *PTEN* FISH was correlated with disease outcomes, only homozygous but not hemizygous *PTEN* loss was associated with decreased recurrence-free survival in multivariate models.[17] Thus, it may be that tumors with heterogeneous PTEN protein loss and underlying homozygous *PTEN* gene deletion have outcomes roughly equivalent to cases with homogeneous PTEN protein loss (the majority of which have homozygous deletion). Though larger case numbers than were included in the current study will be required to formally address this hypothesis, this would suggest that it may be useful to perform reflexive FISH in the case of heterogeneous PTEN protein loss by immunohistochemistry (14% of total cases in current cohort) to determine whether there is underlying homozygous *PTEN* gene deletion. The FISH could be guided by the immunohistochemistry to focus on areas with protein loss, increasing the sensitivity of the assay in this way.

There are a number of limitations of the current study. Though both FISH and immunohistochemistry were performed on the same tissue microarrays, analysis of all tissue microarray cores was not technically feasible for both methods in all cases and correlation between the two assays was carried out on a tumor-by-tumor rather than core-by-core basis for most cases. Thus, some of the disagreements between FISH and immunohistochemistry likely came about because of tumor heterogeneity, where different areas of the same tumor were being analyzed by each assay, and standard section analysis of a subset of cases largely bears this out. In addition,

the gold standard for assessing *PTEN* gene status is not clear at this point. Though FISH can detect larger deletions, which are the most common mechanism of loss in prostate cancer, it will miss smaller deletions, as well as indels and missense mutations, which may inactivate the gene. Thus, in cases where the same tumor tissue was analyzed, it is impossible to know the true cause of the apparent discrepancies between FISH and immunohistochemistry without using a third methodology such as sequencing to examine for gene alterations that would be missed by FISH (these studies are ongoing in separate cohorts currently). Finally, owing to the relatively small numbers of discordant cases overall, it was not feasible to do a meaningful analysis comparing FISH and immunohistochemistry for prediction of prognosis in these cases, to determine which assay is a better prognostic tool.

In conclusion, in a large multi-institutional cohort of prostate tumors, our immunohistochemistry assay for PTEN loss shows the highest specificity and sensitivity for *PTEN* gene deletion reported for an immunohistochemistry assay to date. These data strongly suggest that immunohistochemistry is a cost-efficient method to screen for PTEN loss in prostate tumors, requiring ~$100 and a single 4 μm tumor section for assay performance. In cases with ambiguous PTEN immunohistochemistry results or heterogeneous PTEN protein loss, reflexive *PTEN* FISH may be a useful confirmatory test. This inexpensive, automated, and analytically validated immunohistochemistry assay has already been used to demonstrate the association of PTEN loss with lethal prostate cancer in a large population-based cohort in multivariable models.[18] Ultimately, its portability will enable the performance of clinical validation studies on a large number of additional cohorts, credentialing PTEN as a prognostic and potentially predictive biomarker in diverse clinical settings.

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Disclosure/conflict of interest

TLL has received research funding from Ventana Medical Systems and JAS has consulted for CymoGen Dx, LLC.

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Original contribution

ERG and PTEN status of isolated high-grade PIN occurring in cystoprostatectomy specimens without invasive prostatic adenocarcinoma☆,☆☆

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Summary High-grade prostatic intraepithelial neoplasia (HGPIN) is widely believed to represent a precursor to invasive prostatic adenocarcinoma. However, recent molecular studies have suggested that retrograde spread of invasive adenocarcinoma into pre-existing prostatic ducts can morphologically mimic HGPIN. Thus, previous molecular studies characterizing morphologically identified HGPIN occurring in radical prostatectomies or needle biopsies with concurrent invasive carcinoma may be partially confounded by intraductal spread of invasive tumor. To assess ERG and PTEN status in HGPIN foci likely to represent true precursor lesions in the prostate, we studied isolated HGPIN occurring without associated invasive adenocarcinoma in cystoprostatectomies performed at Johns Hopkins between 2009 and 2014. Of 344 cystoprostatectomies, 33% (115/344) contained invasive prostatic adenocarcinoma in the partially submitted prostate (10 blocks/case on average) and were excluded from the study. Of the remaining cases without sampled cancer, 32% (73/229) showed 133 separate foci of HGPIN and were immunostained for ERG and PTEN using genetically validated protocols. Of foci of HGPIN with evaluable staining, 7% (8/107) were positive for ERG. PTEN loss was not seen in any HGPIN lesion (0/88). Because these isolated HGPIN foci at cystoprostatectomy are unlikely to represent retrograde spread of invasive tumor, our study suggests that *ERG* rearrangement, but not PTEN loss, is present in a minority of potential neoplastic precursor lesions in the prostate.

1. Introduction

High-grade prostatic intraepithelial neoplasia (HGPIN) is widely considered the main precursor lesion to invasive prostatic adenocarcinoma [1–4]. HGPIN is characterized by a proliferation of atypical luminal cells with nuclear and nucleolar enlargement within ducts and acini with an intact basal cell layer [3,4]. Architecturally, HGPIN may show a tufted, micropapillary or flat growth pattern. Though HGPIN is more

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commonly seen in prostates with invasive prostatic adenocarcinoma than those without invasive tumors, meta-analyses have found that the short-term risk of subsequent diagnosis of invasive carcinoma after an initial diagnosis of HGPIN on needle biopsy is not markedly elevated over baseline unless HGPIN is multifocal [5]. Historically, the evidence supporting HGPIN as a precursor lesion for invasive adenocarcinoma has largely been based on its cytologic resemblance to, and frequent association with, invasive tumors [1,2]. In addition, common molecular features have been reported for invasive adenocarcinoma and HGPIN. Of these, the presence of *ERG* gene rearrangement in HGPIN has been one of the most compelling findings. *ERG* or other *ETS* gene family members are rearranged in approximately 50% of invasive prostatic adenocarcinoma cases from patients of European descent, resulting in over-expression of ERG protein [6,7]. In more than 20 prior studies, *ERG* gene rearrangement has been reported to occur in HGPIN associated with invasive cancers, and *ERG* status of the invasive tumor and the adjacent HGPIN are frequently concordant, suggesting a likely clonal relationship between the two lesions [8–28]. Though these data do not prove that HGPIN is a precursor lesion to invasive adenocarcinoma, they are consistent with this hypothesis.

Recently, however, there is increasing recognition that HGPIN may have considerable morphologic overlap with another intraductal lesion in the prostate that has a markedly different natural history. Intraductal carcinoma of the prostate (IDC-P) is widely regarded to commonly result from retrograde intraductal spread of a pre-existing high-grade invasive adenocarcinoma [29,30]. Though the current strict morphologic definition of IDC-P is designed to preclude over-diagnosis of HGPIN as IDC-P, an unavoidable consequence of this specificity is that some true cases of IDC-P are likely underdiagnosed as HGPIN [29,30]. Two recent studies have highlighted this pitfall. Patients with atypical intraductal lesions that fail to meet morphologic criteria for diagnosis of IDC-P (and thus may currently be diagnosed as HGPIN) have a substantially increased risk of subsequent diagnosis with high-grade invasive carcinoma [28]. These data suggest that a wider morphologic spectrum of intraductal proliferations than are currently included in the definition of IDC-P may, in fact, represent retrograde spread of invasive carcinoma rather than true precursor lesions [14,21,28]. In addition, a recent study of ERG-positive intraductal lesions (some resembling HGPIN and some more recognizable as intraductal carcinoma) that were associated with nearby invasive carcinoma demonstrated identical *ERG* rearrangement breakpoints in the HGPIN-like and intraductal lesions and the concurrent invasive adenocarcinomas [31]. Further, the presence of heterogeneous PTEN loss in the invasive tumor with homogeneous PTEN loss in the intraductal and HGPIN-like lesions in these cases strongly suggests that the HGPIN-like and intraductal carcinoma lesions actually represent late-stage ductal colonization by the invasive tumor in at least some cases.

If morphologically identified HGPIN may not always be a precursor lesion in prostate cancer, but may in some cases represent later-stage retrograde spread of adjacent invasive carcinoma, then many prior studies of molecular changes in HGPIN are likely confounded [32]. Because the prevalence of *ERG* gene rearrangement and PTEN loss in HGPIN have only been studied in radical prostatectomies (which invariably harbor concurrent invasive tumor) or in biopsies (where the presence of concurrent invasive tumor is uncertain) may have inadvertently included cases of intraductal spread of invasive carcinoma masquerading as HGPIN [8–28,32]. Thus, the prevalence of these genetic alterations in true precursor lesions to invasive prostate carcinoma remains unclear. To address this, we studied a series of cystoprostatectomy specimens with isolated HGPIN in the absence of concurrent sampled invasive adenocarcinoma that are likely to represent true precursor lesions. Using genetically validated immunohistochemistry assays, we demonstrate that ERG expression occurs in a minority of isolated HGPIN lesions, while PTEN loss is extremely uncommon at this early stage in tumorigenesis.

2. Materials and methods

2.1. Patient and tissue selection

This study, including tissue collection and immunohistochemistry (IHC) staining, was approved by Johns Hopkins Institutional Review Board. A search of the Johns Hopkins Pathology database between 2009 and 2014 for cystoprostatectomy specimens performed for urothelial carcinoma without concurrent reported prostate cancer was made. At Hopkins, gross sectioning of the prostate in cystoprostatectomy cases is geared towards detecting significant prostate carcinomas and urothelial carcinoma involving the prostate and prostatic urethra. In general, 10 blocks of prostate are submitted, focusing on the posterior peripheral zone, with 1–2 separate blocks submitted to examine the prostatic urethra and transition zone. Submitted sections comprise around 30% of the total prostate volume in most cases, unless a gross lesion is detected, in which case the prostate is entirely submitted.

A total of 229 cystoprostatectomy specimens were retrieved from the surgical pathology archives, and all of the prostate slides were reviewed by one pathologist (C.L.M.) to select cases with HGPIN. HGPIN was defined as a proliferation of atypical luminal cells with crowding, stratification and/or irregular spacing, involving ducts and acini. These lesions, generally visible at low power, showed one of the following architectural patterns: tufting, micropapillary, cribriform or flat [3,4]. Enlargement of the nuclei and nucleoli, with nucleoli visible at 20× magnification was required. One to three separate blocks containing HGPIN were selected for each case. Some cases had multiple foci of HGPIN identified within each block (range, 1-3), with a minimal distance of 4 mm between individual foci required to consider the foci separate. Atypical intraductal lesions as described previously were not observed in any cystoprostatectomy specimen [28].

2.2. Immunohistochemistry

One hematoxylin and eosin (H&E)–stained section to verify presence of HGPIN on deeper sections of the block and two 4 μm sections were prepared for immunostaining. ERG immunostaining was performed by using a mouse monoclonal antibody (clone 9FY, Biocare Medical). In brief, following deparaffinization and rehydration, antigen unmasking was done by EDTA buffer (pH 8.0) for 45 minutes. Endogenous peroxidase activity was blocked by incubation in dual endogenous hydrogen peroxidase and alkaline phosphatase enzyme blocker solution for 5 minutes at room temperature. After washing with PBS with Tween 20, non-serum protein block was applied for 5 minutes (ULTRA V Block, Thermo Scientific). Then the primary antibody was allowed to react in dilution of 1:50 for 45 minutes at room temperature. After washing in phosphatase-buffered saline, a horseradish peroxidase–labeled polymer (UltraVision Quanto Detection System HRP DAB, Thermo Scientific) was then applied for 10 minutes at room temperature. Peroxidase was visualized by DAB (3,3′-diaminobenzidine tetrahydrochloride) chromogen. Slides were counterstained with hematoxylin, dehydrated, and mounted.

PTEN IHC was performed as previously described on the Ventana Discovery Ultra automated staining platform utilizing CC1 antigen retrieval buffer (Roche-Ventana Medical Systems, Tucson, AZ) for 32 minutes at 100°C, followed by incubation with a rabbit anti-human PTEN antibody (Clone D4.3 XP; Cell Signaling, Danvers, MA; 1:75 dilution) at 36°C for 32 minutes, followed by the Optiview HRP multimer secondary detection system [33,34].

PIN4 immunostaining in a subset of cases was performed using a prediluted antibody cocktail for p63, cytokeratin 903 and AMACR (Zeta Corporation, Sierra Madre, CA) on the Ventana Benchmark Ultra (Ventana-Roche) automated immunostainer.

2.3. Immunohistochemistry scoring

Nuclear ERG protein was visually scored using a previously validated dichotomous scoring system by 2 pathologists (C.L.M. and T.L.L.) [35]. All glands on the standard slide were scored once they met morphologic criteria for HGPIN, based on side-by-side comparisons with a hematoxylin- and eosin-stained section. Staining for nuclear ERG was assessed in comparison with stromal endothelial cell staining, which provided an internal positive control for ERG in each section. Staining for ERG was considered positive if any lesional cells showed nuclear positivity, even those with somewhat weaker staining when compared with endothelial cells, and negative if no lesional cells were positive.

PTEN immunohistochemistry was blindly scored using a previously genetically validated dichotomous scoring system [33,34,36] by 2 pathologists (L.G. and T.L.L.). A tissue core was considered to have PTEN protein loss if the intensity of cytoplasmic and nuclear staining was markedly decreased or entirely negative across >10% of tumor cells compared to surrounding benign glands and/or stroma, which provide internal positive controls for PTEN protein expression. This simple dichotomous scoring system has been shown to be highly correlated with underlying homozygous genetic deletion of *PTEN* [36].

3. Results

A search of the Johns Hopkins Pathology database revealed that a total of 344 cystoprostatectomies were performed at the Johns Hopkins Hospital from January of 2009 to December of 2014. Of these, 114 specimens had an incidental diagnosis of prostate carcinoma and one cystoprostatectomy was performed for a prostatic stromal sarcoma, and all of these were excluded from the study. Among the 229 cystoprostatectomy specimens where the prostate was reported as free of cancer, 32% (73/229) had identifiable HGPIN and comprised the study cohort. The age of the 73 patients ranged from 46 to 81 years with a median of 66 years. Between 1 and 3 tissue blocks containing HGPIN were sampled for each case, resulting in the inclusion of 133 separate HGPIN foci, varying from 1 to 3 foci for each case.

From 133 HGPIN foci in 73 cystoprostatectomy specimens, 110 HGPIN foci (110/133; 83%) in 61 cystoprostatectomy specimens (84%) met inclusion criteria for the study after performing deeper levels for IHC. The 23 excluded HGPIN foci included 22 foci where the HGPIN focus was no longer present on deeper recuts performed for IHC and 1 case where a small focus of atypical glands, suspicious for carcinoma, appeared on deeper levels. Of the 61 cystoprostatectomy specimens included in the study, 24 (39%) had multifocal HGPIN defined as presence of HGPIN on multiple slides.

Overall, ERG IHC was positive in 7% (8/107) of individual HGPIN foci (Figs. 1-3). Three HGPIN foci (3% or 3/110) were uninterpretable due to weak staining of internal control endothelial nuclei. Interestingly, no specimen had more than one focus of ERG-positive HGPIN, thus a total of 13% (8/61) of individual cystoprostatectomy specimens contained an ERG-positive HGPIN focus (Fig. 3). The rate of ERG positivity was not significantly different among cases with HGPIN present on only one slide (5/37 or 13.5%) versus cases with multifocal HGPIN present on multiple slides (3/24 or 12.5%). In order to confirm that the ERG-positive foci did not represent occult infiltrating cancer with a HGPIN-like morphology, 4 of the positive foci with smaller HGPIN glands were assessed for presence of basal cells using PIN4 immunostaining cocktail. All evaluated foci (4/4; 100%) were positive for basal cell markers, p63 and high-molecular-weight cytokeratin (Figs. 2 and 3).

PTEN immunohistochemistry was interpretable on 80% (88/110) of the HGPIN foci remaining on unstained deeper levels on 60 individual cystoprostatectomy specimens. None of the HGPIN foci (0/88) showed PTEN loss (Fig. 1).

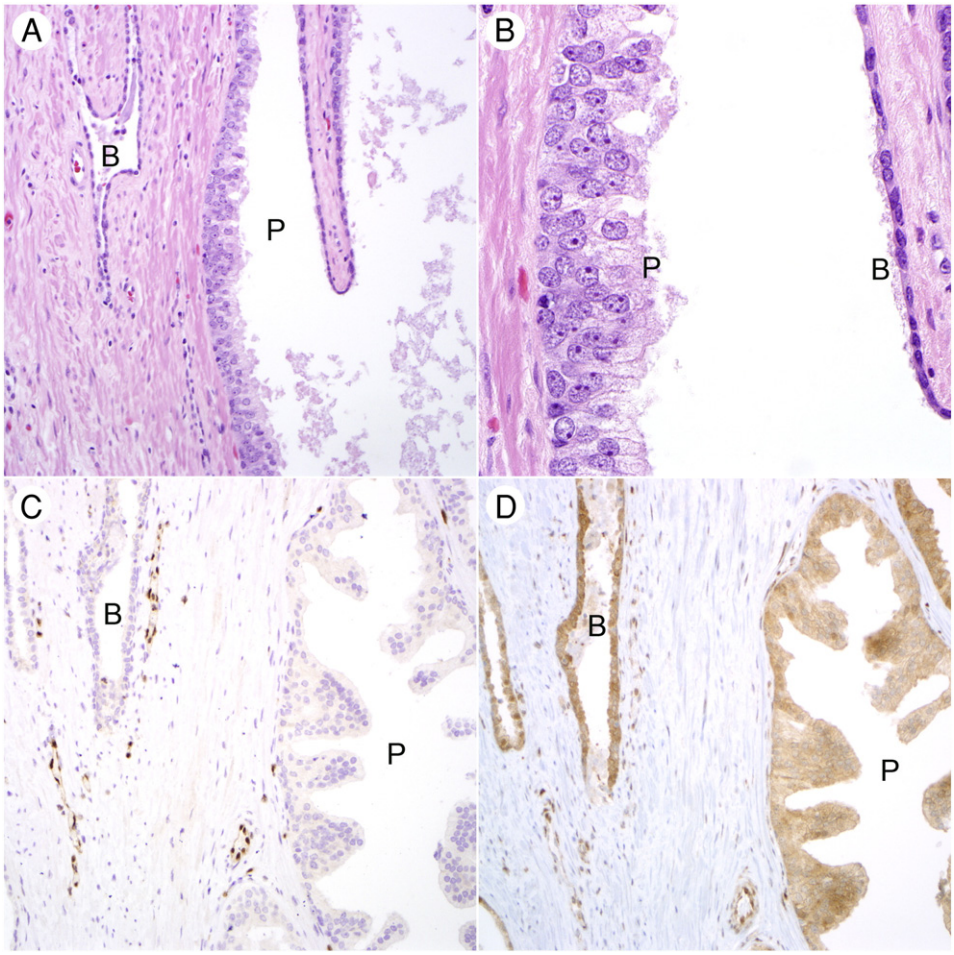

Fig. 1 A, Representative HGPIN lesion (P) from radical cystoprostatectomy with adjacent benign glandular epithelium (B) (200× magnification). B, prominent nucleoli are apparent in HGPIN cells (P) compared to adjacent benign luminal cells (B) (630× magnification). C, Immunostaining for ERG is negative in HGPIN (P) and benign (B) glands, with positive staining in adjacent endothelial nuclei as an internal positive control (200× magnification). D, PTEN immunostaining is intact in HGPIN (P) lesion and adjacent benign (B) glands (200× magnification).

4. Discussion

While a number of histological lesions referred to by various names have been described previously as potential prostate cancer precursor lesions [1], intraductal dysplasia (later referred to as high-grade prostatic intraepithelial neoplasia) was first described with detailed morphological criteria as a potential pre-malignant lesion by McNeal and Bostwick in 1986 [2]. Over the years, a combination of morphological, epidemiological and molecular evidence has been used to support the hypothesis that HGPIN is a precursor to invasive carcinoma [4]. The cytologic similarity between the atypical luminal epithelial cells in HGPIN and invasive carcinoma and the frequent presence of HGPIN adjacent to microinvasive foci of carcinoma ("PINATYP") have long been used to link the two lesions [37,38]. In prior cystoprostatectomy series, men with incidental invasive carcinomas are more likely to have HGPIN compared to those without carcinoma (100% *vs* 63%) [39,40] and the vast majority of tumors occur in a background of multifocal HGPIN (91%) [41]. Further, in autopsy series, the prevalence of HGPIN increases dramatically with age just as prostate cancer does, and a higher age-related prevalence of HGPIN is seen in ethnic groups with a higher prevalence of prostate cancer [42]. Finally, as an isolated finding in needle biopsy specimens, multifocal HGPIN is associated with an increased risk of cancer in subsequent biopsies [5].

The molecular evidence that HGPIN is a precursor lesion to prostate cancer has consisted mainly of molecular alterations in common between invasive adenocarcinoma and HGPIN. Discrete molecular lesions, such as *GSTP1* methylation [43,44] and telomere shortening [45,46] are seen in HGPIN as well as in the majority of adenocarcinomas. Similarly, chromosomal copy number alterations involving chromosome 8p [47,48] and 8q24 [49–51] have been reported in both HGPIN as well as invasive adenocarcinomas. However, perhaps the most convincing alteration reported in both HGPIN and invasive adenocarcinoma involves *TMPRSS2-ERG* gene fusions. Present in nearly half of prostate carcinomas and extremely specific to this tumor type, the prevalence of *ERG*

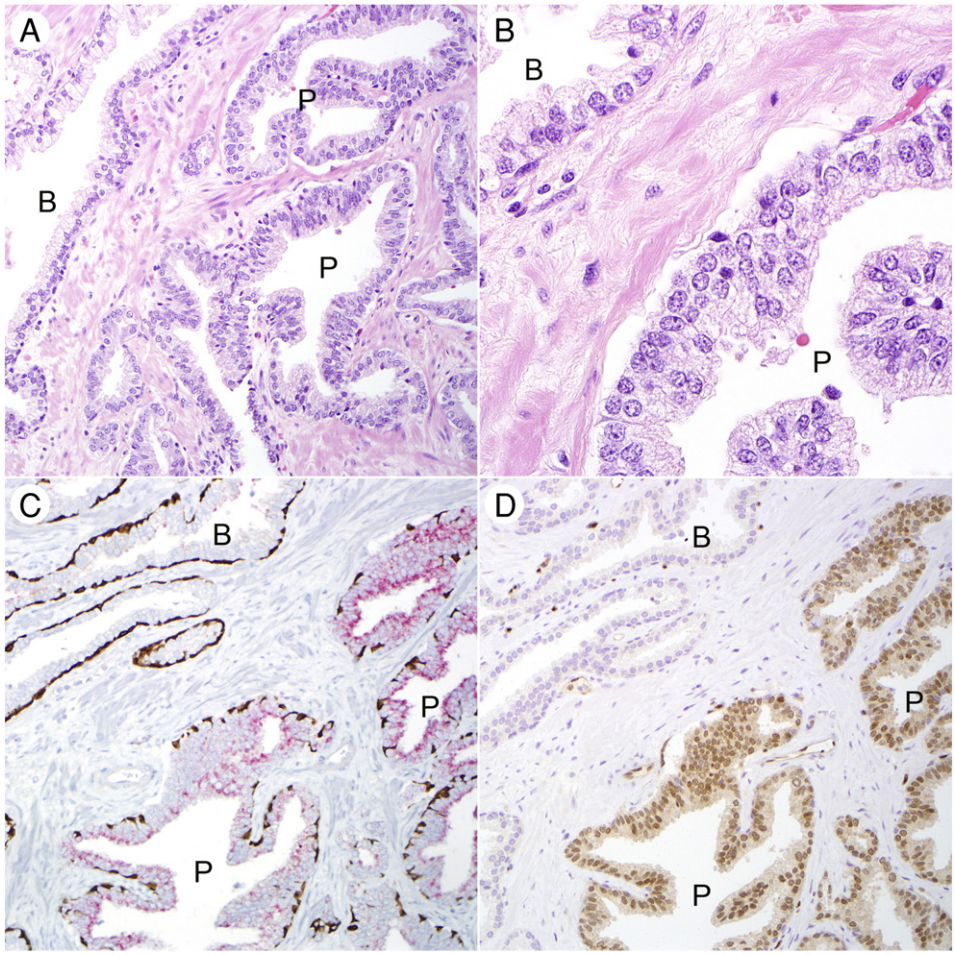

Fig. 2 A, Representative HGPIN lesion (P) from radical cystoprostatectomy with adjacent benign glandular epithelium (B) (200× magnification). B, Prominent nucleoli are apparent in HGPIN cells (P) compared to adjacent benign luminal cells (B) (630× magnification). C, Immunostaining with PIN4 cocktail for high-molecular-weight keratin and p63 demonstrates positively staining basal cells (brown) in both benign (B) and HGPIN (P) glands (200× magnification). Racemase positivity (red) is seen in HGPIN lesion. D, ERG is expressed in nuclei of HGPIN (P) lesion but is negative in adjacent benign (B) glands (200× magnification).

fusions has been repeatedly documented in HGPIN over the past decade. Altogether, our literature review retrieved at least 21 independent studies of *ERG* fusion rates in HGPIN, with over 1100 separate HGPIN lesions queried for *ERG* fusion by FISH, RT-PCR or IHC (Table) [8–28]. Examining either radical prostatectomy specimens or needle biopsies with HGPIN sampled with or without concurrent invasive adenocarcinoma, these studies have found evidence of *ERG* rearrangement in a median of 17% of HGPIN lesions (range, 0%-36%). Because 20/21 of these prior studies utilized radical prostatectomy specimens or needle biopsies with HGPIN and concurrent or subsequent invasive carcinoma, many examined the concordance between the two lesions, and this concordance has generally been high, especially when the HGPIN and invasive carcinoma are located in close proximity to one another (Table). These data have been used to support the argument that HGPIN is a precursor to invasive prostate cancers.

Although the morphologic, epidemiologic and molecular data presented above support a close or even clonal relationship between HGPIN and invasive carcinoma, these data do not help us to discern the temporal or evolutionary relationship between these two lesions. HGPIN could evolve into invasive adenocarcinoma in some cases; however, the possibility that HGPIN could represent late-stage retrograde spread of invasive adenocarcinoma into pre-existing benign ducts would be equally consistent with all of the data summarized above [32]. Ultimately, multiple molecular alterations must be simultaneously examined in HGPIN and adjacent carcinoma to determine whether there is a clonal relationship between the two, and if so, whether one lesion likely gave rise to the other. In a recent study that used *ERG* breakpoint analysis and *PTEN* gene deletion to begin to address this question, it appears that at least a subset of lesions meeting morphologic criteria for HGPIN may have evolved from (rather than into) adjacent invasive carcinoma [31].

Thus, it is likely the case that morphologically identified HGPIN may actually represent a spectrum of intraductal lesions in the natural history of prostate cancer. While some

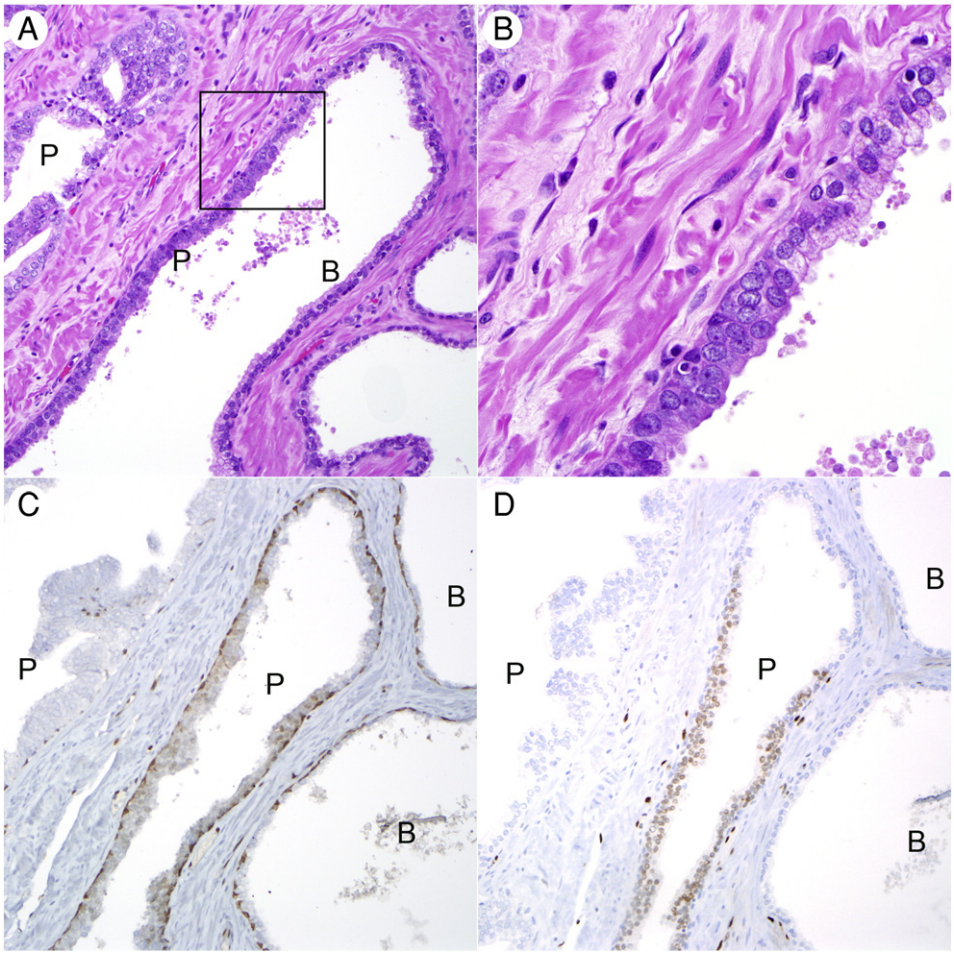

Fig. 3 A, Representative HGPIN lesions (P) from radical cystoprostatectomy with adjacent benign glandular epithelium (B) within the same gland. B, High-power image of boxed region from panel A demonstrates prominent nucleoli in HGPIN cells compared to adjacent benign luminal cells (200× magnification) (630× magnification). C, Immunostaining with PIN4 cocktail for high-molecular-weight keratin and p63 demonstrates positively staining basal cells (brown) in both benign (B) and HGPIN (P) glands. Racemase positivity (red) is absent in this HGPIN lesion (200× magnification). D, Immunostaining for ERG is positive in luminal cells from one of two HGPIN (P) glands and negative and negative in adjacent benign (B) luminal cells within the same gland. An adjacent HGPIN lesion (P) is negative for ERG in the same field (200× magnification).

HGPIN lesions may precede carcinoma and have the capacity to evolve into invasive tumors, others may represent intraductal spread of previously invasive tumor. Because of this, many previous molecular studies of HGPIN are likely confounded since nearly all studies of HGPIN have been performed using radical prostatectomy specimens (which all contain invasive tumor) or needle biopsies (where the existence of concurrent invasive tumor is unknown) [32]. Indeed, because many of the morphologically identified HGPIN lesions in these previous studies are adjacent to invasive tumors, it is likely that at least some of the lesions studied represent retrograde spread of invasive tumors. Thus, inclusion of these cases may make the prevalence of cancer-related molecular alterations in HGPIN look artificially high.

This confounding issue could also potentially explain the results of the few studies that found that the presence of cancer-associated molecular alterations in HGPIN on needle biopsy, such as presence of *ERG* gene rearrangement, is associated with a higher risk of developing subsequent invasive cancer [17,25,32]. Indeed, if the rate of ERG positivity in true precursor HGPIN is low, it is possible that the group of ERG-expressing HGPIN lesions was relatively enriched for retrograde intraductal spread of concurrent unsampled invasive tumors compared to the ERG-negative HGPIN group. This scenario would also explain a higher rate of subsequent invasive cancer in these ERG-positive HGPIN cases when the prostates were resampled by needle biopsy.

To begin to understand the true prevalence of molecular alterations in HGPIN, it is necessary to study HGPIN occurring in the absence of invasive carcinoma, as these are likely to represent true precursor lesions. Though autopsy specimens would be ideal for these studies, poor tissue preservation in autopsies makes identification of the characteristic cytologic features of HGPIN difficult. Here, we studied prostate tissue from cystoprostatectomies performed for urothelial carcinoma where the submitted prostate tissue did not contain prostatic

Table Summary of studies on ERG status in HGPIN

Reference	% ERG+HGPIN	Method	Tissue	% with concurrent invasive cancer	Geographic relationship between HGPIN and invasive cancer	Concordance between HGPIN and invasive cancer for ERG status
Cerveira, 2006 [8]	21% (4/19)	RT-PCR	RP	100% (19/19)	NS	NS
Perner, 2007 [9]	19% (5/26)	FISH	RP (TMA)	100% (26/26)	100% of ERG+ HGPIN in "tight proximity" to cancer	80% (4/5) for ERG+ HGPIN
Furusato, 2008 [10]	14% (2/14)	RT-PCR	RP	100% (14/14)	36% (5/14) adjacent; 64% (9/14) distant	NS
Mosquera, 2008 [11]	16% (23/143)	FISH	RP, Bx	87% (124/143)	NS	73% (91/124)
Carver et al., 2009 [12]	10% (4/10)	FISH	RP (TMA)	100% (10/10)	adjacent	100% (10/10)
Han, 2009 [13]	15% (5/33)	FISH	RP (TMA)	100% (33/33)	Adjacent = <3 mm; away = >3 mm	75% (15/20) for HGPIN adjacent to ERG+ cancer; 0% (0/10) for HGPIN away from ERG+ cancer
Han, 2010 [14]	0% (0/16)	FISH	RP	100% (16/16)	>3 mm	NS
van Leenders, 2011 [15]	52% (11/21)	IHC	Bx	90% (19/21)	Same slide	95% (18/19)
Yaskiv, 2011 [16]	29% (5/17)	IHC	Bx	100% (17/17)	71% (12/17) immediately adjacent to invasive carcinoma	100% (5/5) for ERG+ HGPIN
Gao, 2012 [17]	36% (59/162)	FISH	Bx	37% (61/162) with subsequent invasive carcinoma	NS	NS
He, 2012 [18]	5% (5/94)	IHC	Bx	38% (36/94) with subsequent invasive carcinoma	NS	NS
Tomlins, 2012 [19]	18% (12/68)	IHC	Bx	22% (15/68)	Separate core	40% (6/15)
Liu, 2013 [20]	22% (4/18)	IHC	Bx	72% (13/18)	Separate core	NS
Lotan, 2013 [21]	13% (5/39)	IHC	RP	100% (39/39)	23 PIN <3 mm from PCa and 16 PIN >3 mm from Pca	57% (17/30)
Teng, 2013 [22]	7% (2/29)	IHC	RP (TMA)	100% (29/29)	NS	100% (2/2) for ERG+ HGPIN
Teng, 2013 [23]	6% (4/69)	IHC	RP (TMA)	100% (69/69)	NS	NS
Verdu, 2013 [24]	0% (0/10)	IHC	RP (TMA)	100% (10/10)	"Distant"	80% (8/10)
Park, 2013 [25]	11% (51/461)	IHC	Bx	37% (170/461) with subsequent invasive carcinoma	NS	NS
Taris, 2014 [26]	18% (10/57)	IHC	RP (TMA)	100% (57/57)	NS	NS
Lee, 2015 [27]	27% (12/45)	IHC	Bx	20% (9/45)	Same core	100% (9/9)
Morais, 2015 [28]	0% (0/19)	IHC	Bx	0% (0/19)	NA	NA

Abbreviations: HGPIN, high-grade prostatic intraepithelial neoplasia; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; RT-PCR, reverse-transcription polymerase chain reaction; RP, radical prostatectomy; TMA, tissue microarray; Bx, biopsy; NS, not specified; PCa, prostate cancer; PIN, prostatic intraepithelial neoplasia; NA, not available.

adenocarcinoma. Though one important weakness of our study is that the prostate in cystoprostatectomy specimens is not submitted in totality for histologic examination at our institution, our prostate sampling procedure in these specimens is likely adequate to detect most significant prostate tumors (with at least 30% of total prostate volume submitted in most cases). In addition, we could clearly exclude the presence of invasive tumor within 3 millimeters of the HGPIN lesion examined. Ultimately, this study will need to be confirmed in a series of cystoprostatectomy specimens containing HGPIN where the prostate is entirely submitted for histologic examination to exclude the possibility of occult invasive cancer in some cases. However, overall, it is highly likely that the HGPIN lesions identified in our cystoprostatectomy study did not result from

retrograde spread of invasive tumor (which typically happen in the context of clinically significant, high-stage tumors [29]), but are instead representative of a spectrum of true precursor lesions in the prostate.

In the current study, we found that 7% of these precursor HGPIN lesions express ERG protein by immunohistochemistry, a proven surrogate for the presence of *ERG* gene rearrangement. This prevalence of ERG expression in HGPIN is at the low end of the range reported in previous large studies of radical prostatectomy and needle biopsy specimens. In one of the largest studies utilizing 143 radical prostatectomies and biopsies, *ERG* rearrangement (measured by FISH) was found in 16% of lesions [11]. In the largest study of biopsies alone (from a prostate cancer prevention trial, GTx Protocol G300104), ERG expression (by IHC) was seen in 11% (51/461) of cases overall [25]. Interestingly, most of the tumors found subsequent to HGPIN in this study were low-grade (Gleason score 6) tumors that may not have had the capacity for retrograde intraductal spread. Thus, the rate of ERG positivity in this study is not significantly different from what we observed in the current study using cystoprostatectomies. However, another large study of prostate biopsies using FISH found that 36% (59/162) of HGPIN lesions showed *ERG* rearrangement [17]. Thus, it is possible that the inadvertent inclusion of some cases of intraductal retrograde spread of tumor may have artificially inflated the prevalence of ERG expression reported in some prior HGPIN studies.

Given that *ERG* rearrangement can occur in a minority of isolated HGPIN lesions, it is unlikely that the presence of ERG-positive PIN will be useful to distinguish true HGPIN from retrograde intraductal spread of cancer. However, PTEN may be useful in this context. We have previously reported that PTEN loss occurs at a high frequency in IDC-P, and can be seen in a majority of IDC-P lesions occurring with concurrent invasive adenocarcinoma [21,28]. In contrast, we found that PTEN loss rarely occurs in HGPIN lesions, either occurring in radical prostatectomies (adjacent or distant from invasive adenocarcinoma) or in needle biopsies where HGPIN was an isolated finding. Here, we add to these data by showing that PTEN loss does not occur at a detectable frequency in isolated HGPIN occurring in cystoprostatectomy specimens. These data add additional support to the concept that PTEN loss in HGPIN or other atypical intraductal lesions may have a high positive predictive value for the presence of concurrent invasive adenocarcinoma, suggesting that these cases should get additional and very close follow-up to exclude this possibility.

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